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Masters of Science by Research

The Survival Strategies of Microphytobenthos: Behaviour and Physiology

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Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution. To the best of my knowledge and belief this thesis contains no material previously written by another person except where due acknowledgement is made in the text.

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Abstract

Microphytobenthos (MPB) need photoadaptive strategies to survive the highly dynamic light environment in which they reside. They are able to adjust their photosynthetic activity by physiological regulation or behaviourally by migrating vertically through the sediment. This study investigates the effects of the time of day on the vertical migration of benthic diatoms at two sites near Hobart, Tasmania using a pulse amplitude modulation fluorometer (Water PAM; Walz, Effeltrich) to measure chlorophyll fluorescence. Chlorophyll *a* content and maximum quantum yield (F_v/F_m) were used to examine the profiles of microalgal biomass and photosynthetic properties within sediment cores, both diurnally and over a 12 month experimental period. The results show a seasonal pattern of chlorophyll *a* biofilm development, with maximum values attained in summer at Pipe Clay Lagoon and in spring at Browns River. A greater amount of biomass was observed in the muddier sediment at Browns River with a sharper peak of chlorophyll *a* compared to the gradual incline then decline at Pipe Clay Lagoon. F_V/F_M values changed throughout the day with the cells more quenched at midday than sunrise while experiencing the highest illumination.

Xanthophylls can provide photoprotection to MPB cells by cycling between epoxide and de-epoxide forms to dissipate excess light energy as heat. The second part of the study examined the xanthophyll cycle in microphytobenthos on tidally exposed sediment at Browns River. The goal of this work was to examine whether microphytobenthos at Browns River used the xanthophyll cycle as a physiological defence against photoinhibition during a natural light-dark cycle (day-night). A High Pressure Liquid Chromatography (HPLC) system was used as a pigment separation technique followed by pigment detection using a photodiode array and quantification against pure pigment standards. A pulse amplitude modulated (PAM) fluorometer was used to determine the chlorophyll fluorescence and assess photosynthetic performance in terms of maximum PSII quantum yield (F_v/F_m), non-photochemical quenching and E_k in the field. Changes in PAM fluorescence and xanthophyll: chlorophyll *a* ratios suggests that MPB were under physiological stress at noon. The results indicate that the MPB cells exposed to light at the surface migrated deeper into the sediments to replenish the

epoxide form of their xanthophylls. Overall the result suggests that MPBs utilise both behavioural and physiological strategies to survive in the dynamic intertidal environment.

This research highlights the importance of the photoadaptive strategies of MPB in a changing light environment with particular reference to the need for more than one strategy. This research on MPB ecology helps to form a more accurate picture on survival strategies while it underlines the fact that previous research has shown inconsistencies. Further research is needed in this area, particularly in the southern hemisphere, to lessen these inconsistencies and build on current knowledge.

Chapter 1

The Survival Strategies of Microphytobenthos: Behaviour and Physiology

Microphytobenthos

Microphytobenthos (MPB) is the term given to microalgae communities, which colonise the surface of intertidal and shallow subtidal sediments world wide (Brotas *et al.* 2007). MPB are comprised of chlorophytes, euglenids and cyanobacteria with the dominate group in most estuarine ecosystems being Bacillariophyceae (diatoms). Over the last few decades there has been an increased interest in these benthic microalgae that inhabit shallow marine areas, and their function in the benthic community (Miles and Sundback 2000). Most scientific interest in MPB has been driven by the increasing recognition of its importance for primary production, it being one of the major contributors to productivity of estuaries and shallow coastal waters (Serodio *et al.* 2007). Intertidal sediments are some of the most productive natural ecosystems on earth, despite the extreme conditions of periodic exposure to air, water movement and other physicochemical changes and their restricted habitat on the sediment surface (Honeywill *et al.* 2006). They comprise the base of the ecosystem as food for invertebrates, fish and wading birds (Cohn and Disparti 1994; Heip *et al.* 1995). They also increase sediment stability by excreting extracellular polymeric substances (EPS) (Consalvey *et al.* 2004) with the critical erosion threshold of sediments observed to increase by 300% in the presence of EPS producing diatoms (de Deckere *et al.* 2001).

Species composition of biofilms, within the estuarine environment, varies spatially and seasonally (Oxborough *et al.* 2000). Benthic communities are comprised of both free-living motile algae (epipelic) and algae attached to sand grains that can sometimes move very slowly (episammic diatoms) (Wolfstein *et al.* 2000). The layer they form is called a biofilm, which exhibits high rates of primary production, plays an

important role in intertidal sediment dynamics and influences nutrient exchanges at the sediment interface (Haubois *et al.* 2005).

The intertidal areas in which the MPB reside are dynamic, and subject to hydrodynamic processes such as waves, currents and tidal exposure (Mitbavkar and Anil 2004). These algal communities are also subject to regularly alternating periods of air exposure and turbid submersion (Miles and Sundback 2000). MPB are frequently subjected to rapid changes in light exposure, including the sudden exposure to high levels of sunlight after withstanding long periods in the dark. This occurs frequently with the ebb and flow of the tides throughout the day (Serodio *et al.* 2005). Intertidal biofilms can be exposed to high temperatures and irradiances (exceeding 2000 μmol photons $m^{-2}s^{-1}$), in particular when tidal emersion periods correspond with solar maxima in summer (Perkins *et al.* 2001).

Approximately 45% of the photosynthesis on earth is from aquatic environments (Falkowski and Raven 2007). Photosynthesis supplies the primary source of organic matter to support the ecosystem, therefore the rate of photosynthesis controls the overall biomass and productivity of the ecosystem and constraints the overall biological flow of energy throughout earth (Falkowski and Raven 2007). Photosynthesis in intertidal environments is limited to the narrow illuminated layer of the surface (Kelly *et al.* 2001) which is known as the euphotic zone. This area of intense biological activity establishes chemical and biological gradients over a microscale (Kelly *et al.* 2001). The depth of 1% light level (euphotic zone) in sediments varies from 0.1 to 13.2 mm depending on granulometry and organic content of the sediment (Saburova and Polikarpov 2003). The light penetration rate of sediment decreases rapidly with increasing sediment depth (Ichimi *et al.* 2008). Generally the depth of the sediment euphotic zone is positively correlated with increasing median grain size (Pinckney and Zingmark 1993). In organic rich mud flats, cells are generally limited to the upper most 1 mm, with 90% of the light attenuated in the top 400 μm, therefore restricting photosynthetic activity (Consalvey *et al.* 2004). During the day time exposure period the chlorophyll *a* maximum in muddy sediments generally occurs in the top 200 μm (Wiltshire 2000, de Brouwer and Stal 2001, Kelly *et al.* 2001). The depth to which cells are distributed is affected by sediment type and light penetration, depth of anoxic layer, wave action and species specific migration

speed (Consalvey *et al.* 2004). However a substantial part of the biomass of diatoms may also be found in deeper layers due to high tide turbulence (Mitbavkar and Anil 2004) and bioturbation.

Survival Strategies

MPB have the capacity to adjust their photosynthetic activity, in response to changing ambient light, through physiological regulation of absorbed light energy and behaviourally through active control of light absorption (Serodio *et al.* 2006). Behavioural regulation is the relationship between light and cell position controlled by vertical migration, which optimises light availability whilst avoiding damaging high irradiances (Jesus *et al.* 2006). Although the exposure to light is controlled at the individual cell level, by the movement of cells within the vertical light gradient, the effects are expressed at the community level through variation in the biofilm biomass present in the euphotic zone (Serodio *et al.* 2006). Physiological regulation includes non photochemical quenching of energy by diversion of excess light energy away from photosystem reaction centres using processes such as the xanthophyll cycle (Jesus *et al.* 2006). Other survival strategies in the low light areas include adaptation to long periods in the dark at low metabolic cost and with negligible degradation of pigments, uptake of dissolved organic compounds or transformation from a vegetative to a resting stage (Mitbavkar and Anil 2004).

Behavioural strategies: Migration

The rhythmic appearance of colouration in the sediment of intertidal areas by the movement of microphytobenthos was first recorded in 1907 by Fauvel and Bohn. Migration (movement of an organism from one area to another) has been documented as a predictable pattern in benthic diatom communities (Consalvey *et al.* 2004). Migration is seen as an advantage as it offers safety from tidal currents, as wind induced waves cause pressure on these environments (Consalvey *et al.* 2004). Low light conditions remove any photosynthetic advantage for cells to be at the sediment surface, from which they could

be potentially eroded. Furthermore, the migration of cells down into the sediment has recently been linked to nutrient uptake and cell reproduction (Saburova and Polikarpov 2003). Downward migration acts to reduce disturbance and grazing and increases nutrient availability (Decho 2000) with subsurface nutrient reserves possibly playing an important role (Kingston 2002). This ability to move is an advantage and can be used to explain the success of diatoms in this environment (Consalvey *et al.* 2004).

Most of the photosynthetic organisms found in intertidal areas are motile, which enables them to migrate to the surface during daylight and into the sediment when the area is immersed by the tide (Honeywill *et al.* 2006). While both epipelic and episammic taxa can move, only the epipelic species move sufficiently fast to undergo rhythmic migrations (Round 1971). Motile epipelic diatoms represent an important component of microphytobenthos and dominate the community in fine and cohesive sediments (Haubois *et al.* 2005). The excretion of extracellular polymeric substances (EPS) plays a role in movement and the ability to adhere to sediment surfaces (de Brouwer and Stal 2001). It has been suggested that EPS strands adhere to the sediment whilst connected to free transmembrane structures which are moved along the raphe through their interaction with actin bundles (Consalvey *et al.* 2004).

Diatom motility is affected by environmental variables such as light, temperature, oxygen/water content and varies with the time of day. Most migrations have geographic and species specific idiosyncrasies, however the most widely described pattern involves an emergence at daybreak with low tide or the exposure period and a downward movement prior to or at high tide or darkness (Consalvey *et al.* 2004). Round and Palmer (1965, 1966) reported general descriptions of the migration of diatoms which included effects such as; cells move to the sediment surface during daylight hours when the surface is exposed to the tides, prior to submersions cells move away from the surface. Individual species come to the surface at different times and may arrive in a distinct order and remain for differing times. Species may appear at the sediment surface for a short period before leaving but may return, showing a bimodal presence at the surface. Cells can exhibit different migration patterns during morning and afternoon tides. These observations by Round and Palmer (1965, 1966) are likely to be time, location and taxon

specific, with localised events also altering patterns (i.e. rain) and the same location can have different migratory patterns.

The depth of migration is also location, grain size and taxon-specific. Saburova and Popikarpov (2003) reported 4.2 cm as the maximal depth in areas of clay sublayers and 8 cm in coarse sands. Other work has shown a depth of 3 mm for diatoms in mudflats and sandy areas (Pinckney *et al.* 1994; Mitbavkar and Anil 2004). Viable cells have also been observed 10 cm or more below the surface of sandy sediments (MacIntyre *et al.* 1996), however this may be due to sediment re-working and grazing rather than migration. It has been hypothesised that a subtle micro-migration at the surface also takes place, with individual species cycling within the biofilm during the upwards phase of migration occurring at a μm scale rather than 1 mm scale, this would support the observation that photoinhibition has rarely been recorded in the microphytobenthos (Consalvey *et al.* 2004).

As the optimum irradiance for photosynthesis varies with latitude and season due to different photoacclimation states, variations of migration behaviour is different in winter and summer (Mitbavkar and Anil 2004). The time of low tide and the diatom populations are two main factors responsible for these seasonal differences in migration (Mitbavkar and Anil 2004). Montani *et al.* (2003) observed strong seasonal and inter-annual variability in the occurrence and development of MPB assemblages. A study undertaken in Japan also observed that concentration of chlorophyll *a* in surface sediment (0-5 mm) measured hourly varied with time of day in every month studied (Koh *et al.* 2007). The daily mean values of chlorophyll *a* and phaeopigments in this study at the surface varied greatly, increasing from summer to winter and decreased in early spring (Koh *et al.* 2007). A study by Mitbavkar and Anil (2004) showed that in summer, low tide exposure in the morning with higher irradiance, low tidal amplitude and a dominant population of epipelic diatoms triggered the upward migration of diatoms in contrast to winter. Migratory behaviour does not appear to be related to time of day, rather to duration of light exposure which is in turn dependent on the irradiance, time of low tide and the existing diatom populations (Mitbavkar and Anil 2004).

No universal diatom migration pattern has yet been described, as their behaviour is species and location specific, however light and tides are likely to be the driving forces

(Consalvey *et al.* 2004), although there is some disagreement over their level of importance. Some studies have shown the same migratory pattern in different species regardless of light regime. Other studies have shown that migration is not maintained if the sample is not wetted (Consalvey *et al.* 2004). Single explanations for migration behaviour would appear to be an oversimplification as many factors are likely to be involved including; endogenous (aerotaxy, geotaxy, phototaxy), light (diurnal changes, weather), tide, CO_2 limitation, disturbance (grazers, rain), nutrient limitation or other factors not yet determined (Consalvey *et al.* 2004) (Fig 1).

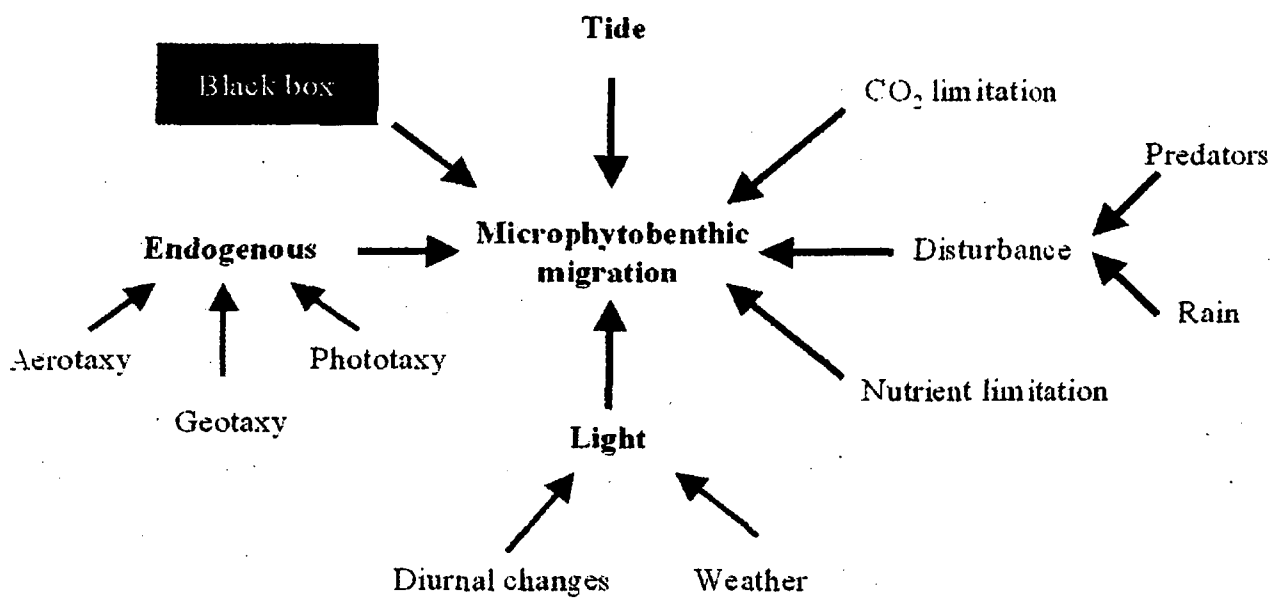


Figure 1. The driving forces of Microphytobenthic migration. The black box represents unknown cues (Consalvey *et al.* 2004).

Microphytobenthic cells are adapted to a highly variable light environment, as cells at the surface are exposed to potentially damaging levels of irradiance during day time exposure periods. However, several studies have shown *in situ* microphytobenthos do not show photoinhibition, which may be due to migration (Barranguet *et al.* 1998; Kromkamp *et al.* 1998). Phototaxis is the directional response to light, with positive phototaxis towards light and negative phototaxis away. Round and Palmer (1966) and Nultsch and Hader (1988) proposed that there is a short period of phototaxy with cells

moving to sediment surface during higher light intensities followed later by negative phototaxy or the strengthening of geotaxy. Therefore, Paterson (1986) concluded that light drives migration based on the orientation of the cells as they emerge. Irradiance at depths is less than the incident irradiance at the surface and is dependent on the attenuation characteristics of the sediment. Kingston (1999) reported that diatoms rise to the surface out of phase with the tides on bright days in winter when covered by clear water. The necessity of light for maintenance of vertical migration is supported by observations that algae do not migrate to the surface during night time low tides or during the day under an opaque canister (Palmer and Round 1965, 1967). Mitbavkar and Anil (2004) also observed upward movement during high tide coverage suggesting an overriding influence of light over tides.

Physiological strategies - Xanthophyll cycle

In excessive light it is essential for microalgae to avoid the over–excitation of their photosynthetic reaction centres, as this can result in permanent damage (Eskling *et al.* 1997). The dangers of high light are intensified under conditions of environmental stress such as low temperature and desiccation which are often encountered in intertidal areas. Physiological regulation through non-radiative dissipation of excess energy is an important short term process for the photoprotection of Photosystem II against light induced damage (Lavuad *et al.* 2004). Absorption of sunlight for photosynthesis is accomplished by light harvesting pigment protein complexes (LHC) that are associated with reaction centres (Muller *et al.* 2001). Photoprotective dissipation of excess light energy is attributed to rapid modifications within the LHC of PSII, leading to non photochemical chlorophyll *a* fluorescence quenching (NPQ) (Lavuad *et al.* 2004). Absorption of sunlight that exceeds a plants capacity for CO_2 fixation results in an increase of the thylakoid ΔpH that is generated by photosynthetic electron transport (Muller *et al.* 2001). The change in pH within the thylakoid lumen is an immediate signal of excessive light that triggers the feedback regulation of light harvesting qE (energy dependent quenching) (Muller *et al.* 2001). The control by lumen pH allows induction or reversal of qE within seconds of a change in light intensity, which in MPB is important

for rapid light changes such as passing clouds on a partly sunny day (Muller *et al.* 2001). A decrease in lumen pH induces qE through protonation of PSII proteins and activation of xanthophyll synthesis via the xanthophyll cycle (Muller *et al.* 2001).

The xanthophyll cycle occurs in the thylakoid membranes of all higher plants, ferns, mosses and several algal groups (Eskling *et al.* 1997). There are two variants, the violaxanthin cycle, which is more commonly found in higher plants and the diadinoxanthin (DD) cycle found in some algal groups (Eskling *et al.* 1997). Xanthophylls are carotenoids containing one or more oxygen radicals and are essential for survival and ecological success (Lohr and Wilhem 2001). They provide photoprotection by quenching the excited states of chlorophylls and by the harvesting and efficient transfer of light energy to chlorophylls (Lohr and Wilhem 2001). Virtually all organisms performing aerobic photosynthesis have the capacity to form zeaxanthin or diatoxanthin. In chromophyte algae such as diatoms, dinoflagellates and prymnesiophytes, the xanthophylls diadinoxanthin (DD) and diatoxanthin (DT) function as photoprotective pigments (Fujiki *et al.* 2003). Some microalgae (Xanthophyceae and Chrysophyceae) also posses alternative xanthophyll cycle pigments involving carotenoids, violaxanthin and zeaxanthin (Brown *et al.* 1999).

The DD cycle found in diatoms, involves a rapid and reversible conversion from DD (one epoxide group) to DT (no epoxide group) (Muller *et al.* 2001). This leads to the dissipation of excess energy to non-radiative pathways, decreasing the transfer of captured excitation energy to the PSII reaction centres and thus limiting the amount of photodamage to the photosynthetic apparatus (Serodio *et al.* 2005). Conversions between DD and DT are mediated by reversible light epoxidizing enzymes in the thylakoid chloroplast membrane which utilizes a pH gradient across the lumen membrane (Moisan *et al.* 1998). Under low to high light transitions, DD is de-epoxidised into DT and DD accumulates under low light conditions (Moisan *et al.* 1998). In contrast, DT is epoxidised to DD during high to low light transitions and accumulates during high light conditions. DD de-epoxidation begins rapidly after the onset of high light with the rate being light intensity dependent (Lavuad *et al.* 2004). The activation of the DD cycle is closely followed by the gradual increase in the pool size of the DD pigments (minute-hour order) (Kashino and Kudoh 2003). The reversal of DD cycle pigments (DT to DD)

is also an important process for the recovery of the 'efficiency' of the utilization of light energy (Kashino and Kudoh 2003). The amount of DT synthesized via the DD cycle is correlated with the level of qE (Muller *et al.* 2001). NPQ and DT are linearly related and if DT is not present, NPQ can not occur (Lavuad *et al.* 2004). Under more prolonged, severe light stress qE is replaced by photoinhibitory quenching, a sustained slowly reversible component of NPQ called qI (Muller *et al.* 2001).

Techniques for measuring MPB: photosynthsis, biomass and pigments

Since the first recorded observation of colouration in intertidal sediment there have been numerous studies conducted to investigate MPB using a range of techniques. These include visual observations (Perkings 1960), lens tissue (Eaton and Moss 1966), cover slip (Paterson 1986), light microscope (Paterson 1986), video camera (Sundback *et al.* 1997), remote sensing (Kromkamp *et al.* 1998) and the cryolander method (Whiltshire *et al.* 1997) in which undisturbed sediment cores at depth resolution of 100 μm are cross sectioned to investigate diatom dynamics at the surface (de Brouwer and Stal 2001). The quantitative study of migration of benthic algae has been accomplished using destructive techniques such as sample freezing and sectioning, cell collection with coverslip or lens tissue, to asses short term changes (Serodio *et al.* 1997). Such methods prevent repeat measurements on a sample thus requiring extensive replication and limiting precision and the spatial and temporal resolution of the experiments (Serodio *et al.* 1997). The High Pressure Liquid Chromatography (HPLC) pigment analysis has also been used as a separative method, which makes it possible to isolate chlorophyll *a* which can be used as a reliable index of biomass of microbenthic algae as well as other pigments (Brotas and Plante-Cuny 1998). Recently Pulse Amplitude Modulation (PAM) fluorescence has become a widely used and reliable technique (Consalvey *et al.* 2004).

Fluorescence

PAM fluorometry was originally designed for terrestrial plants but is now applied to aquatic organisms. Serodio *et al.* (1997) were the first to use pulse modulated

fluorescence techniques to investigate benthic sediment biofilms and monitor biomass. Fluorometry allows for rapid, reproducible measurement of meaningful fluorescence parameters *in situ* and *in vivo* (within intact biofilms) at temporal and spatial scales that are relevant to the microphytobenthic ecology (Oxborough *et al.* 2000). One of the major advantages is that the fluorescence signals originate solely from photosynthetic organisms and are unambiguously related to gross photosynthesis (Kolber and Falkowski 1993). The technique also examines the sample in real time which with the non destructive nature minimises artefacts caused by experimental manipulation. PAM fluorometry also continuously measures fluorescence signals from a variety of platforms potentially allowing for widely expanded temporal and spatial resolution of phytoplankton (Kolber and Falkowski 1993).

- *Chlorophyll a fluorescence*

Diatoms need to maintain photosynthetic activity in a range of light conditions, including the variations of light within a day and between seasons (Ralph and Gademann 2005). There are two photoreactions in all oxygenic photoautophobic organisms, known as Photosystem I (PSI) and Photosystem II (PSII) (Kolber and Falkowski 1993). In PSII water is photochemically oxidised forming O_2 while simultaneously generating electrons and protons (Kolber and Falkowski 1993). The flow of electrons through PSII is indicative of the overall rate of photosynthesis (Maxwell and Johnson 2000). In PSI light energy is used to further transfer the electrons to terminal acceptors. At physiological temperatures, fluorescence emanates almost entirely from PSII. The theoretical basis for relating fluorescence to photosynthesis is that excitation energy delivered to PSII absorbed by chlorophyll molecules can undergo one of three fates: photochemistry (drives photosynthesis), dissipated as heat (NPQ) or re-emitted as light (fluorescence) (Maxwell and Johnson 2000) (Fig 2). These three factors occur in competition, therefore an increase in one will result in a decrease of yield of the other two (Maxwell and Johnson 2000). PSII is considered to be the most vulnerable part of the photosynthetic apparatus to light induced damage therefore harm to this will often be the first manifestation of stress (Maxwell and Johnson 2000).

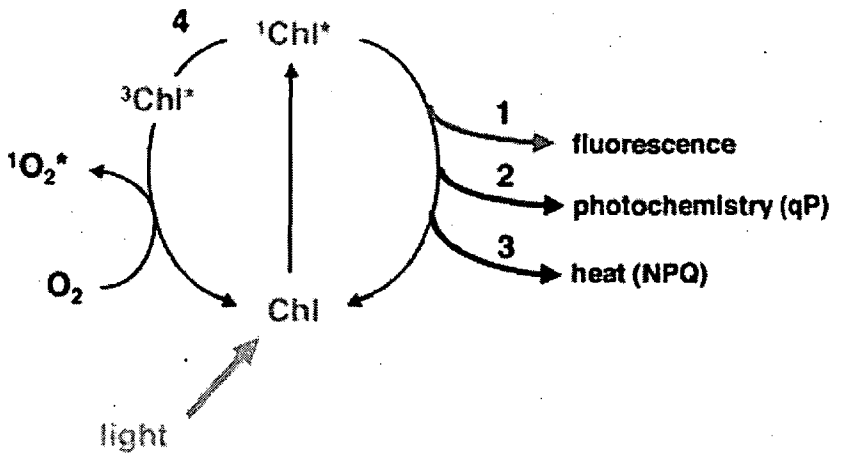


Figure 2. Possible fates of excited Chlorophyll (Muller *et al.* 2001).

The majority of chlorophyll forms a light harvesting complex at PSII, which is connected with a reaction centre via smaller peripheral antennae (Laisk *et al.* 1997). The reaction centres in PSII consist of a primary electron donor, a special chlorophyll *a* molecule (designated P_{680}) and a primary electron acceptor (a quinone designated Q_A) (Kolber and Falkowski 1993). A change in yield of chlorophyll fluorescence was first observed by Kautsky *et al.* in 1960 when transferring photosynthetic material from the dark into the light (Maxwell and Johnson 2000). This increase however, is explained as a consequence of reduction of electron receptors in the photosynthetic pathway, downstream from PSII, notably plastiquinone and in particular Q_A (Maxwell and Johnson 2000). Upon exposure to light, photons may be absorbed by the photosynthetic pigments and the excitation energy transferred to the reaction centre leading to charge separation. During this charge separation P_{680} is oxidised to P_{680}^+, and Q_A is reduced to Q_A^- (Kolber and Falkowski 1993). Once PSII absorbs light and Q_A has accepted an electron it is not able to accept another until it has passed the first onto a subsequent electron carrier (Q_B). During this time the reaction centre is said to be closed. Only once P_{680}^+ is re-reduced and Q_A^- is re-oxidised can the energy of another absorbed photon be used for the next photochemical electron transfer (Kolber and Falkowski 1993). Upon reduction Q_B physically dissociates from the reaction centre complex to become part of the plastiquinone (PQ) pool (Kolber and Falkowski 1993).

When the proportion of closed reaction centres leads to an overall reduction in efficiency of photochemistry there will be a corresponding increase in fluorescence (Maxwell and Johnson 2000). In the dark when Q_A is completely oxidised, fluorescence is at a minimal level. Under ambient light the fluorescence yield increases as Q_A becomes reduced reaching a maximum, when all reaction centres are closed (Kolber and Falkowski 1993). Thus in its simplest form, the relative change in quantum yield of fluorescence reflects the redox state of Q_A and the relationship between photochemistry and fluorescence is inverse (Kolber and Falkowski 1993).

- *PAM fluorometry*

The spectrum of fluorescence is different to absorbed light, with the peak of fluorescence emission being of longer wavelength than that of absorption. Therefore fluorescence yield can be quantified by exposing a sample to light of defined wavelength and measuring the amount of light re-emitted at longer wave lengths (Maxwell and Johnson 2000). In a modulated system the light source used to measure fluorescence is modulated (switched on and off at high frequency) and the detector is tuned to detect only fluorescence excited by the measuring light. Therefore the relative yield of fluorescence can be measured in the presence of background illumination and in the presence of full sunlight in the field (Maxwell and Johnson 2000). Fluorometers use different intensity lights to manipulate the photosynthetic apparatus which in turn emit different amounts of fluorescence (Ralph and Gademann 2005). PAM fluorometers firstly use a weak measuring light to determine the proportion of closed PSII reaction centres, without inducing photosynthesis. The fluorescence emitted (fluorescence yield) as a result of this measuring light only, is called minimum fluorescence (Fo if the sample is dark adapted or Ft if it is light adapted) (Ralph and Gademann 2005). The second light source used to assess photosynthetic activity is a saturating pulse which is used to close all PSII reaction centres resulting in a substantially greater fluorescence emission (Fig 3.) (Ralph and Gademann 2005). This is the maximum fluorescence (Fm in dark adapted samples or Fm' in the light adapted). The difference between the minimum and maximum fluorescence is known as Fv (variable fluorescence) and can be used to determine the maximum efficiency of light utilisation at PSII (Honeywill *et al.* 2002).

The final light is actinic light used to induce photosynthesis with a range up to 2000 μmol photons $m^{-2}s^{-1}$ (Ralph and Gademann 2005).

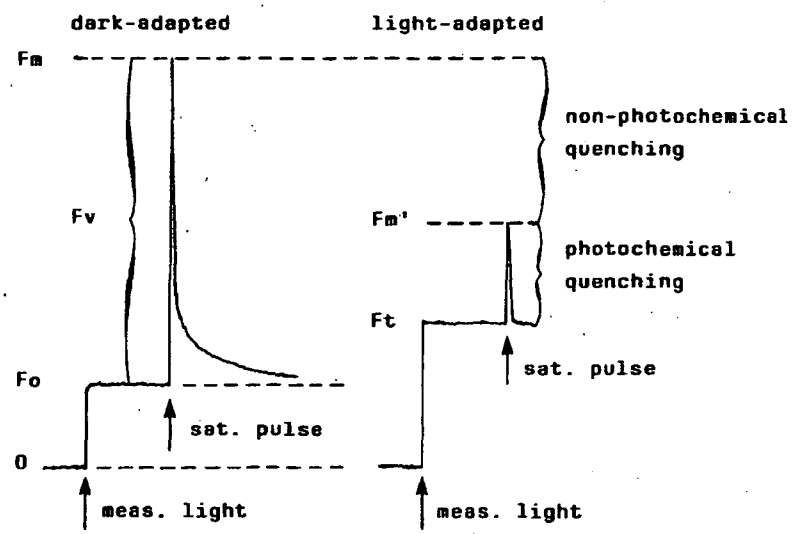


Figure 3. Principle of saturation pulse quenching analysis (WALZ 1999).

Whether a sample is light or dark adapted before the reading will influence the fluorescence yield. Dark adaptation allows PSII reaction centres to open, electron transport chain to be oxidised, photoprotective mechanisms to be relaxed (xanthophyll cycle) and the ΔpH gradient to be depleted (Ralph and Gademann 2005). Measurement of the photosynthetic efficiency of PSII can be derived from the minimum and maximum values of florescence including maximum quantum yield (F_V/F_M when dark adapted) and effective quantum yield ($\Delta F/F_M$' when light adapted). The effective quantum yield is calculated by (F_M'-F)/F_M'. A commonly used symbol for effective quantum yield is ΔF/Fm', however there are numerous symbols used in the literature including ∂F/Fm, Φ_{PSII}, and F_q'/F_M. Light adapted measurements of quantum yield are lower than the maximum due to the inherent impact of non-photochemical quenching reducing the light adapted yield (Ralph and Gademann 2005). The absorbed light leads to closure of reaction centres elevating the F_O level. Hence the variable fluorescence and F_V/F_M are lower in the light adapted state. F_V/F_M is described as a dimensionless ratio, it is the

maximum quantum yield for stable charge separation at PSII and is a measure of the maximum photochemical efficiency and is calculated as $(F_M-F_O)/F_M$ (Villareal 2004).

A rapid light curve (RLC) measures the effective quantum yield as a function of irradiance (Ralph and Gademann 2005). Light curves present photosynthetic capacity and the potential activity over a range of ambient light intensities (Ralph and Gademann 2005). They provide a reliable assessment of photosynthetic activity and reflect its immediate short term light history (Ralph and Gademann 2005). A RLC is conducted by using 10 seconds of actinic light at each of 8 light steps; it reflects the current light acclimation status of the sample (Villareal 2004). RLCs are also known as rapid P-I curves and instant light response curves (Ralph and Gademann 2005). They are influenced by the long term pre history (i.e. low light and high light adapted) and can reflect the relative condition throughout diel and tidal cycles (Ralph *et al.* 2002).

RLCs have three distinct regions; light limited, light saturated and photoinhibited. With low irradiances, photosynthesis is limited by the light intensity (Ralph and Gademann 2005). The rise of the curve in the light limiting regions (α) is proportional to the efficiency of light capture (effective quantum yield). Minimum saturating irradiance (E_k) is determined by finding the interception of α with the maximum photosynthetic rate (Ralph and Gademann 2005). It is an indicator of the photoacclimation state as irradiance is continuously fluctuating and acclimation takes time, E_k is constantly changing (Sakshaug *et al.* 1997). Under moderate irradiance, the capacity of the electron transport chain limits photosynthesis and the curve reaches a plateau where maximum photosynthetic capacity occurs ($rETR_{max}$). With higher irradiances the curve tends to decline which could be due to photoinhibition or linked to dynamic down regulation of PSII (White and Critchley 1999).

- *Fluorescence quenching*

The increase in chlorophyll fluorescence when the PSII reaction centres are closed is generally followed by a decrease over a few minutes. This phenomenon is called fluorescence quenching and can be explained in two ways (Maxwell and Johnson 2000). Firstly by the light induced activation of enzymes involved in carbon metabolism which is associated with the redox state of Q_A, known as photochemical quenching (qP).

Secondly, at the same time there is an increase in the efficiency with which energy is converted to heat, known as non-photochemical quenching (NPQ) (Maxwell and Johnson 2000). Therefore, fluorescence usually does not follow a simple inverse relationship with photochemistry (Kolber and Falkowski 1993). Photosynthetic organisms have developed strategies to optimize light harvesting at low intensities while minimizing photoinhibitory damage due to excess energy at high light intensities (Lavaud *et al.* 2002). After a period of hours they regulate the quantity and composition of the light harvesting complexes and of a number of other components of their photosynthetic apparatus (Lavaud *et al.* 2002). On a shorter time scale they react to an inbalance between light intensity and photosynthetic capacity by rapid structural modifications within the LHC of PSII. This leads to a decrease in chlorophyll fluorescence yield (Consalvey *et al.* 2004). NPQ dissipates excess energy through a harmless non-radiative pathway (Lavaud *et al.* 2002).

NPQ is induced by the formation of a photon gradient across the thylakoid membrane and is associated with the operation of a xanthophyll cycle, which converts epoxidized to deepoxidized forms of xanthophylls (Lavuad *et al.* 2004).. Serodio *et al.* (2005) observed variations in NPQ upon changes in irradiance were generally followed by the proportional variations in DT content. This was observed under high light, during which the build up of NPQ was closely paralleled by a proportional increase in DT concentration. In diatoms, higher growth irradiances induced larger DD pools, increasing the production of energy dissipating DT under high light and enabling higher NPQ levels (Cruz and Serodio 2008). However, higher NPQ values may also be caused by an increase in photoinhibitory damage to the photosynthetic apparatus (qI) (Cruz and Serodio 2008). A larger DD pool in high light acclimated cultures could also explain the high NPQ values frequently observed in dark adapted samples (Cruz and Serodio 2008).

Diatoms are also characterised by the existence of non-photochemical fluorescence quenching in the dark adapted state, which may result from the establishment of a trans-thylakoid proton gradient in the dark caused by chlororespiration (Ting and Owens 1993; Schreiber *et al.* 1995). MPB are frequently subjected to rapid changes in light exposure, particularly with the tides throughout the day, therefore the maintenance of functional xanthophyll cycle pigments by benthic diatoms is advantageous and may explain high levels of NPQ in the dark (Serodio *et al.* 2005). Dark

NPQ may also be caused by the increased content or activity of the enzyme DD-depoxidase, due to a higher pH required for activation, leading to higher degrees of de-epoxidation independently of the DD pool size (Cruz and Serodio 2008).

HPLC pigment analysis

High Pressure Liquid Chromatography or High Performance Liquid Chromatography (HPLC) is a separation technique used for both quantitative and qualitative analysis. This chromatographic technique, which consists of using small diameter columns, fine particle size and rapid flow rate is now a widespread tool for investigating microalgal pigment compositions (Brotas and Plante-Cuny 2003). The use of HPLC pigment analysis is now increasingly being applied to MPB with the additional advantage of achieving a much better discrimination of degraded pigments (Brotas *et al.* 2007). The scale of pigment analysis expanded rapidly in the 1980s with the development of automated HPLC techniques for pigment separation (Jeffrey *et al.*1999). Not only were accurate quantitative analysis of chlorophylls then available (free of degradation products) but these HPLC methods allowed separation of up to 20 taxonomically useful carotenoids from mixed phytoplankton populations with over 40 pigments being separated in a single run (Jeffrey *et al.*1999). HPLC pigment analysis for taxonomy is based on the premise that different algal classes have specific signature or marker pigments (Li *et al.* 2002). From the whole set of pigments, some pigments are ubiquitous in all algal classes, such as chlorophyll *a* and β carotene, some are exclusive of a particular class or division, such as alloxanthin for Cryptophyta, and the majority are shared between a number of classes, for example fucoxanthin, which is present in Bacillariophyta, Chrysophyceae, Prymnesiophyceae, Raphidophyceae and some Dinophyceae (Brotas and Plante-Cuny 2003). HPLC makes it possible to isolate chlorophyll *a* which can be used as an accurate measure of biomass of microbenthic algae (Brotas and Plante-Cuny 1998).

Summary

Intertidal areas are important ecosystems found world wide. Microphytobenthos constitute an important basis of estuarine and shallow ecosystems food webs, contributing to the stability of sediment and playing a major role in the biogeochemical cycles through the production of oxygen (Brotas *et al.* 2007). MPB need photoadaptive strategies to cope in the dynamically changing light environment of the intertidal zone. It has been 100 years since the first recorded observation was made on the migration of benthic diatoms. Since that time the methodology and technology for investigating microphytobenthos has evolved. Migration is advantageous for benthic diatoms in a dynamic and unstable environment whether to escape intense light and tidal pressures or to increase illumination for photosynthesis. Most authors agree that light and tides are central in the control of migration in microphytobenthic organisms. Physiological regulation through non-radiative dissipation of excess energy is also an important short term process for photoprotection (Lavuad *et al.* 2004). PAM fluorometry and HPLC pigment analysis are seen as effective techniques for studying intertidal communities. Their ability to report photosynthetic parameters and pigments can be used to explain the physiological state of a community at different times of the day and year. This gives valuable insight into the photoadaptive strategies, both behavioural and physiological, used by MPB in a changing environment over short and long time scales.

Chapter 2

Diurnal and Monthly Vertical Profiles of Benthic Microalgae within Intertidal Sediments from Two Tasmanian Sites

Abstract

Intertidal areas in which microphytobenthos (MPB) reside are dynamic with changes in light intensity over a short (tides) and longer time scales (seasonally). The ability of MPB to migrate away or towards the sediment surface to optimise sunlight exposure or avoid excess light is one of the reasons they are so successful in intertidal areas. This study investigates the effects of the time of day on the migration of benthic diatoms at two sites near Hobart, Tasmania, using a pulse amplitude modulation fluorometer (Water PAM; Walz, Effeltrich) to measure chlorophyll fluorescence. Chlorophyll *a* content and maximum quantum yield (F_v/F_m) were used to examine the profiles of microalgal biomass within sediment cores, both diurnal and over a 12 month experimental period. The results show a seasonal pattern of chlorophyll *a* biofilm development, with maximum values attained in summer at the sandy site, Pipe Clay Lagoon, and earlier in spring at Browns River, a muddier site. The muddier site was observed to contain an overall greater level of biomass than the sandy site. The F_v/F_m values demonstrated that the cells were more 'stressed' at midday when sunlight was highest compared to sunrise throughout the year; however a significant seasonal variation was only observed at Browns River. The benthic microalgal community at Browns River and Pipe Clay Lagoon did not clearly demonstrate vertical migration through the sediment, between July 2005 and June 2006. It is likely that the MPB at Pipe Clay Lagoon and Browns River are using photoadaptive strategies in conjunction with a small scale vertical migration below the detection limit of the applied methods.

Introduction

The cyclic, rhythmic appearance of colouration in sediment of intertidal areas was first recorded in 1907 by Fauvel and Bohn (Consalvey *et al.* 2004). This colouration is

caused by the presence of microphytobenthos (MPB), which inhabit the top centimetres of intertidal sediment in estuarine and coastal areas worldwide consisting of chlorophytes, euglenids, cyanobacteria with the dominate group generally being Bacillariophyceae (Kelly *et al.* 2001). Microphytobenthos play a key role in the functioning of estuarine ecosystems by providing the principal source of carbon and moderating carbon flow, food for invertebrates and increasing sediment stability by EPS production (Cohn and Disparti 1994; Heip *et al.* 1995; Underwood and Kromkamp 1999; Middelburg *et al.* 2000; Consalvey *et al.* 2004).

Microphytobenthos reside in areas of steep irradiance gradients with spatially and temporally shifting patterns of tidal exposure resulting in high irradiance at times while at other times very little or no light (Underwood and Kromkamp 1999). Therefore, it can be hypothesized that the ability to move away or towards the surface to optimise sunlight or avoid turbulence is an advantage and is one of the reasons diatoms are so successful in these environments (Consalvey *et al.* 2004). Several studies have shown *in situ* microphytobenthos do not show photoinhibition because of downward migration (Barranguet *et al.* 1998; Kromkamp *et al.* 1998). However, when measuring intact biofilms, the contribution of deeper layers to depth-integrated photosynthesis also plays a role (Forster and Kromkamp 2004; Serodio 2004). The necessity of light for maintenance of vertical migration is supported by observations that algae do not migrate to the surface during night time, low tides or during the day under an opaque covering (Palmer and Round 1965, 1967). Most microalgal migrations have geographic and species specific idiosyncrasies, however the most widely described pattern involves the emergence prior to or at the start of the day time low tide, and a downward movement prior to or at high tide/darkness (Consalvey *et al.* 2004).

Photosynthesis in intertidal environments is limited to the narrow illuminated layer of the surface (Kelly *et al.* 2001). The depth to which cells are distributed is affected by this light penetration but is also affected by sediment type, depth of anoxic layer, wave and current action, species specific speed and other taxa present (Consalvey *et al.* 2004). The depth of migration is location and taxon specific with maximal depths of 4.2 cm in areas of clay sublayers and 8 cm in coarse sands (Saburova and Popikarpov 2003). Migratory behaviour is not solely related to time of day, but also to duration of

light exposure, which is in turn dependent on the irradiance, time of low tide and the existing diatom populations (Mitbavkar and Anil 2004). No universal migration pattern has been described but light and tides are believed to be the driving forces (Consalvey *et al.* 2004).

Here, we investigate the effect of changing light levels over 12 months on the algal abundance of two sites near Hobart, Tasmania using a Water PAM (Walz, Effeltrich). We examine light as the primary determent of the position of MPB in intertidal areas. It is hypothesized that the rapid and gradual changes in light duration and intensity experienced in these areas determines the position of the cells. It is observed how this cell position is affected by the type of sediment, diurnally and seasonally.

Materials and Methods

The migration of benthic diatoms within sediments was examined at two intertidal sites including Pipe Clay Lagoon (42°97'S, 14°732') (Fig.1) and Browns River (42°96'S, 147°51'E) (Fig. 2) in the greater Hobart area (Fig. 3). The total area of Pipe Clay Lagoon is 5.3 km^2 with a total catchment area of approximately 16.5 km^2 (DPIWE 1998). The land surrounding the lagoon is characterised by large rural residential sized holdings with 0.5 km^2 used for marine farming. The lagoon is a large, open, exposed site which is often used for recreation. The average flushing time of the lagoon is estimated to be 1.36 tidal cycles, the maximum depth is 4-5 m and there is a lack of major freshwater inputs (DPIWE 1998). Browns River Catchment covers an area of 59 km^2 with numerous streams, tributaries and sub catchments (MEMS 2003). The sub-catchment closest to the study site is 5.5 km^2 with an average flow rate of 0.035 m^3sec (MEMS 2003). Upstream from Browns River is an industrial site with residential buildings adjacent. Browns River has a large mouth to the sea which is always open. The width of the river is approximately 5 m with a depth of 1.5-2 m. The flushing time of Browns River has not been measured however it is assumed that due to the large mouth into the sea the cycle would be rapid in a similar time to Pipe Clay Lagoon. Browns River is shaded by dry sclerophyll Eucalyptus species on the north bank. Pipe Clay Lagoon and Browns River were sampled once a month, except February, between July and June 2005-2006.

Figure 1. Pipe Clay Lagoon

Figure 2. Browns River

Figure 3. Maps of study sites a) Tasmania b) Browns River 1:125000 c) Pipe Clay Lagoon 1:125000.

On each sampling date the sites were sampled four times between sunrise and sunset, with times varying depending on time of year and day length (Appendix 1. tidal information during sampling times). During each sampling period seven 15 mm diameter Perspex sediment cores were taken; three for fluorescence analysis, three for chlorophyll analysis and one for taxonomic purposes. Cores were always taken from the same area at a location which was never fully exposed during the sampling. The corer was manually pushed into the sediment and stoppered using a rubber bung and then carefully and quickly returned to shore. The core was sliced in 2 mm intervals for the length of the euphotic/oxygenated zone, the size of which varied and was identified through colour differentiation.

Filtered sea water (0.22 μm membrane filter; Pall Supor, New York) was added to each of the 3 replicates to resuspend each 2 mm sediment sample. A pulse amplitude modulated (PAM) fluorometer (Water PAM, Walz, Effeltrich) was used to determine the chlorophyll fluorescence. This technique produces fluorescence parameters *in situ*, *in vivo* (within intact biofilms) and in this study *in vitro*, at temporal and spatial scales that are relevant to the microphytobenthic ecology (Oxborough *et al.* 2000). It also examines the sample in real time minimising artefacts caused by experimental manipulation (Kolber and Falkowski 1993).

To calculate the maximum photosystem II quantum yield (F_v/F_m) the samples were dark adapted for 15 minutes by wrapping the sample jars in foil and placing them in a light proof container. Rapid light curves (RLC) were taken under software control (Wincontrol, Walz) to obtain values for maximum quantum yield (F_v/F_m), Relative electron transport rate (rETRmax), the light utilisation coefficient (α) and the light saturation parameter (E_k) (Ralph and Gademann 2005). A RLC is a light treatment with eight consecutive 10 s of actinic light of increasing intensity light levels; it generates data that reflects the current light acclimation status of the sample (Villareal 2004). Red light emitting diodes (LED) provided the actinic light used in the RLC at levels of 0, 63, 96, 142, 221, 329, 470, 657 and 1093 μmol photons $m^{-2}s^{-1}$. rETR was calculated by multiplying the irradiance by the quantum yield measured at the end of that interval (Genty *et al.* 1989). PAR vs rETR curves were described using the model of Platt *et al.* (1980) and multiple non-linear regression curve fitting techniques on Systat software (v5.2 for Macintosh Systat Inc.). The initial slope of the function is termed α, a measure of the plant cells' ability to utilise light. As the function reaches a plateau, the maximum photosynthetic rate occurs (rETRmax). E_k is calculated from the intercept between rETRmax and α (Falkowski and Raven 2007).

The core samples for chlorophyll analysis sliced in 2 mm intervals and were placed in a light proof container and transported to the laboratory where they were placed in a -80°C freezer until analysed. Chlorophyll was extracted in 10 ml of methanol and measured on a Turner Designs 10AU fluorometer using the acidification method (Holm-Hansen *et al.* 1965). The fluorometer was calibrated against a Chlorophyll *a* standard (Sigma Chemical Co. Missouri).

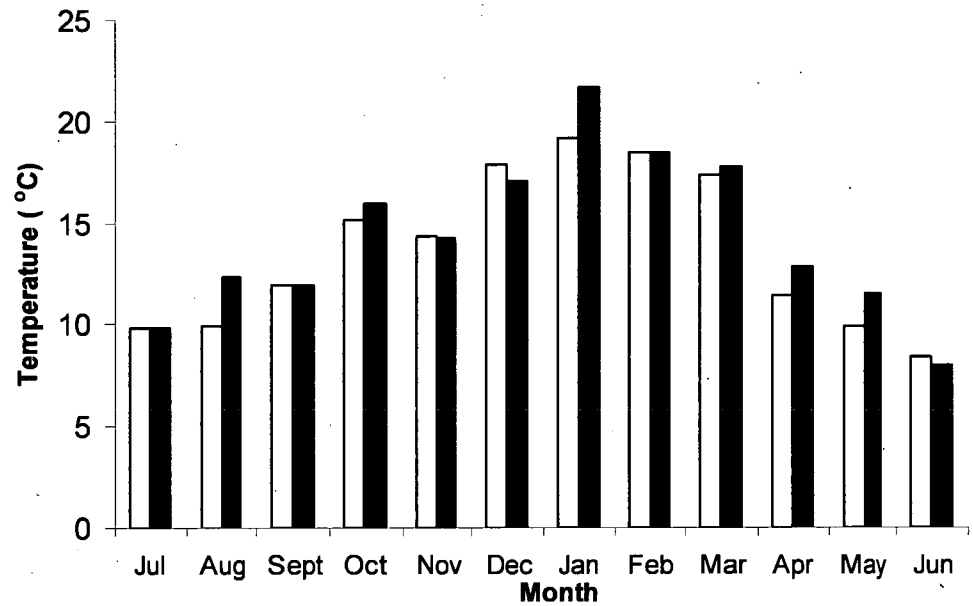
Temperature, salinity, pH, turbidity, and dissolved oxygen were recorded by lowering a Horiba U-10 water quality checker (Horiba, Japan) into the water column and measuring at the sediment interface at noon on each sampling date. A Quantum Scalar Irradiance meter QSL 100/101 (Biospherical Instruments Inc. California) was used to measure ambient light in the water column. The taxonomic samples were examined using light microscopy (Primo Star, Zeiss) and classified with reference to Hodgson *et al.* 1997; John 1983; Taffs 2005 and Witkowski *et al.* 2000.

After examining preliminary results of the four sampling intervals it was concluded that using the two data sets of sunrise and midday, as they reflect the greatest difference in illumination, would be sufficient to explore the diurnal pattern. Statistical analysis was a comparison of two multivariate time series. For each site a record over time of four responses (chlorophyll *a* and F_v/F_m, morning and noon) was documented. These can be compared with regression, but it is necessary to adjust for any serial correlation as the order in which the months occur can not randomised, as it is likely that in addition to any consistent seasonal changes, two months that are closer in time will be more alike than two months separated in time. To test for diurnal and seasonal changes of F_v/F_m and chlorophyll *a*, generalised least squares regression (GLS) models assuming an AR(1) correlations structure to account for serial correlations through time were fit to the data by Restricted Maximum Likelihood (REML) method (Pinheiro and Bates 2000) (appendix 2).

Results

Pipe Clay Lagoon has sandy sediment with 96.56% sand (grain size >63 μm) and 2.59% mud/silt (grain size 2-63 μm) and the MPB was dominated by a combination of *Navicula*, *Cocconeis* and *Achnanthes* species. Browns River has a muddier sediment (33.84% sand, 58.97% mud/silt), and was dominated by *Navicula*, *Cocconeis*, *Nitzschia*, *Amphora* and *Pleurosigma* species. Water temperature and salinity values over the study period were similar at the two sites (Fig. 4). Water temperature increased from a minimum of 9.4°C in winter to 21.7°C in summer. The salinity values were relatively stable ranging from 26 to 35 ppt over the 12 months.

a)



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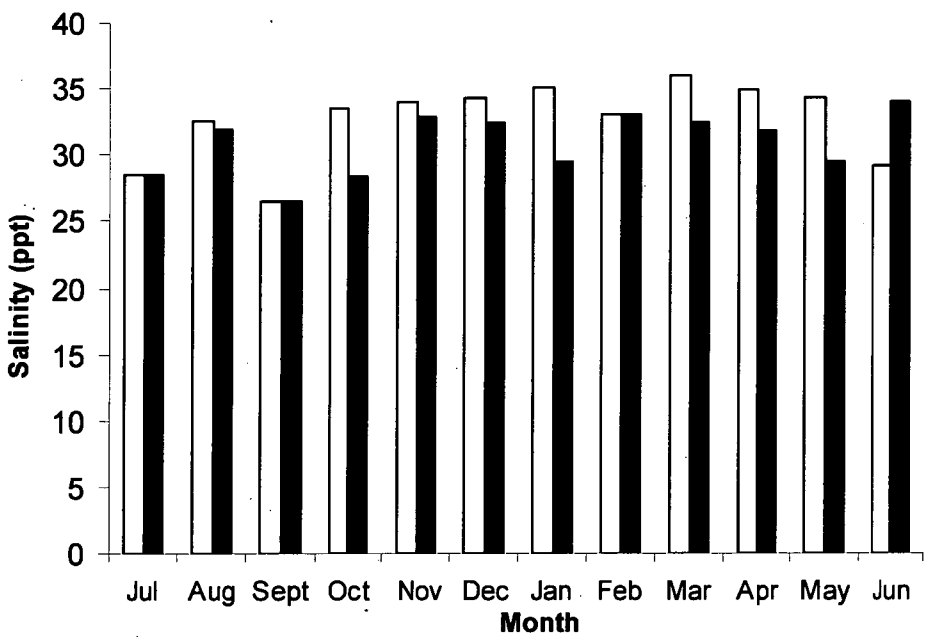


Figure 4. Comparison of Browns River (black) and Pipe Clay Lagoon (white) from July 2005 to June 2006 in a) Water temperature and b) Salinity. Note July, September and February readings taken from the Department of Primary Industries and Water 2004/2005 (unpublished data).

Chlorophyll a

When examining chlorophyll *a* at the two sites there was no evidence of any time of day effect or interaction between time of day and site or season. However, there was strong evidence of a season-site interaction. The two sites showed a significantly different pattern of chlorophyll *a* over the 12 months ($P<0.001$) (Fig. 5). At Pipe Clay Lagoon the chlorophyll *a* biomass of the surface 2 mm at sunrise ranged from 10.7 ± 1.0 mg chl *a* m^{-2} in July to 33.5 ± 3.6 mg chl a m^{-2} in December. There is strong evidence of a seasonal difference at Pipe Clay Lagoon ($P<0.001$) with the surface 2 mm biomass steadily increasing until maximum levels were reached in summer, and then declined to winter. At Browns River the chlorophyll *a* biomass of the surface at sunrise ranged from 6.98 ± 0.4 mg chl *a* m^{-2} in April to 43.55 ± 5.0 mg chl a m^{-2} in October (Fig. 5). Over the 12 month period investigated at Browns River there was a significant difference between the months ($P<0.001$) with a steady increase in chlorophyll *a* to October, when it reached its peak, followed by a steady decline through summer and autumn.

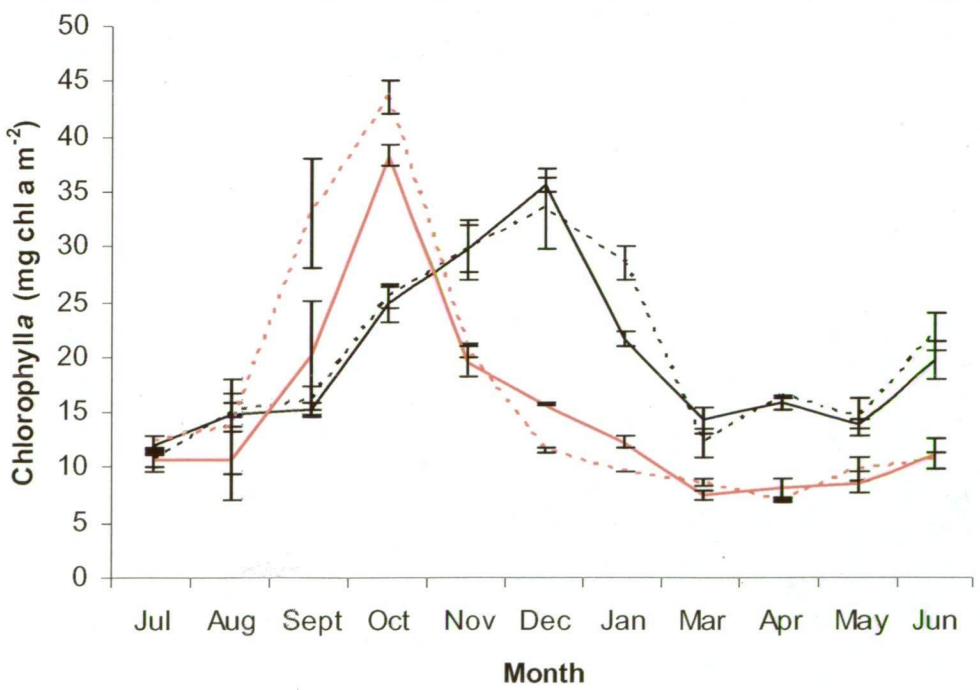
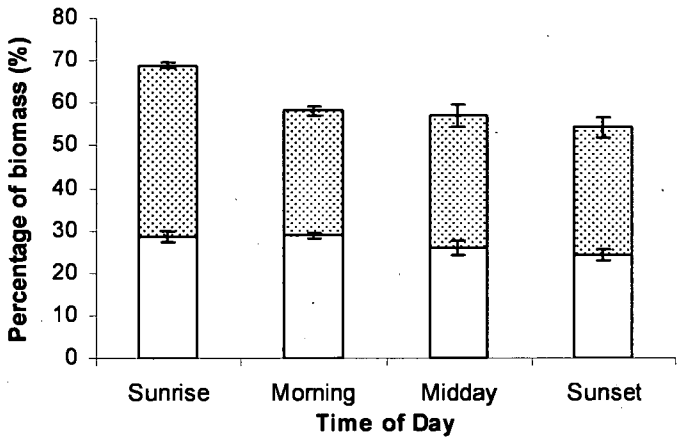


Figure 5. Profiles of Chlorophyll *a* concentrations by month for the surface 2 mm at Pipe Clay Lagoon (black) and Browns River (red) at sunrise (broken) and midday (solid) from July 2005 to June 2006. Values are means ± standard error.

A small change occurred in chlorophyll *a* in the surface 2 mm and the 2-4 mm depth interval at both sites. This trend in biomass was observed for every month studied, as shown in November (Fig. 6). Typically, at Pipe Clay Lagoon (Fig. 6a) the biomass in the surface 2 mm contained approximately 20-40% of the biomass throughout the day. The samples from 2-4 mm had only a slightly lower biomass although this was only significantly different to the surface 2 mm during sunrise in October, April and May and at midday in October, December, January and April ($P<0.05$; data not shown). This trend was also observed at Browns River (Fig. 6b). The only times in which the surface 2 mm biomass was significantly greater than 2-4 mm biomass in Browns River was in January, March and July at sunrise, September, June and July in the morning, during midday in January, and July and April at sunset ($P<0.05$; data not shown). The surface biomass was always greater than the 2-4 mm interval except at Browns River in March at midday and sunset although the difference was not significant ($P>0.05$).

a)



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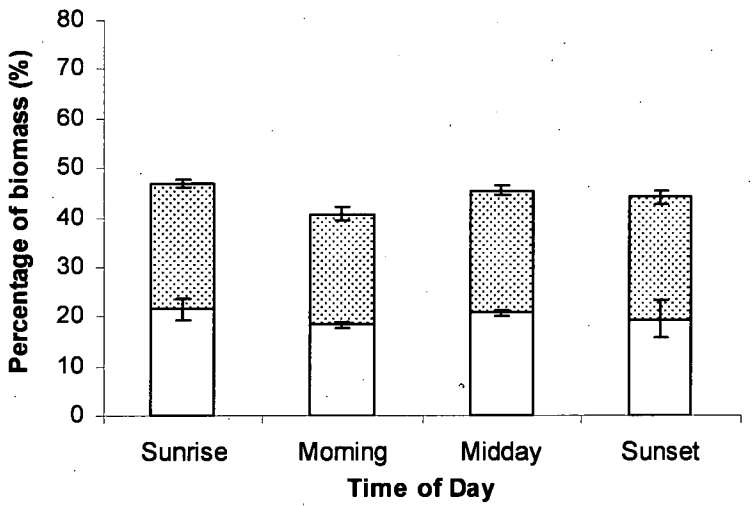


Figure 6. Percentage of the algal biomass in the surface 2 mm (dots) and 2-4 mm (clear) of sediment in November 2005 at a) Pipe Clay Lagoon and b) Browns River. Values are means ± standard error.

Fluorescence

The maximum PSII quantum yield of chlorophyll fluorescence (F_V/F_M) of the surface 2 mm demonstrated a significant relationship with the time of day and a seasonal pattern that differed between the sites (Fig. 7). The values of F_V/F_M were not significantly different between the two sites (P=0.104). Both sites demonstrated a time of day effect

with an overall higher F_V/F_M value at sunrise than at midday ($P<0.001$). At Pipe Clay Lagoon the surface F_V/F_M ranged from 0.42 ± 0.07 in November to 0.57 ± 0.04 in October, although there was no evidence of a seasonal difference ($P=0.514$). At Browns River the F_V/F_M of the surface 2 mm ranged from 0.41 ± 0.02 in January to 0.58 ± 0.08 in September and June (±0.005) (Fig. 7). There was a significant seasonal difference at Browns River with F_V/F_M values lower in summer and higher in autumn and winter ($P<0.001$).

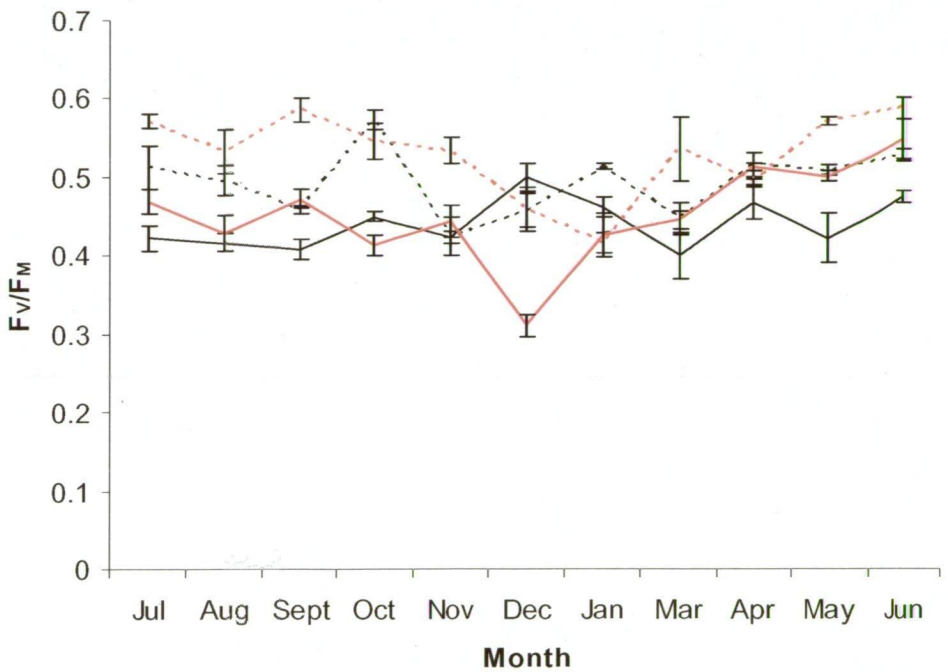
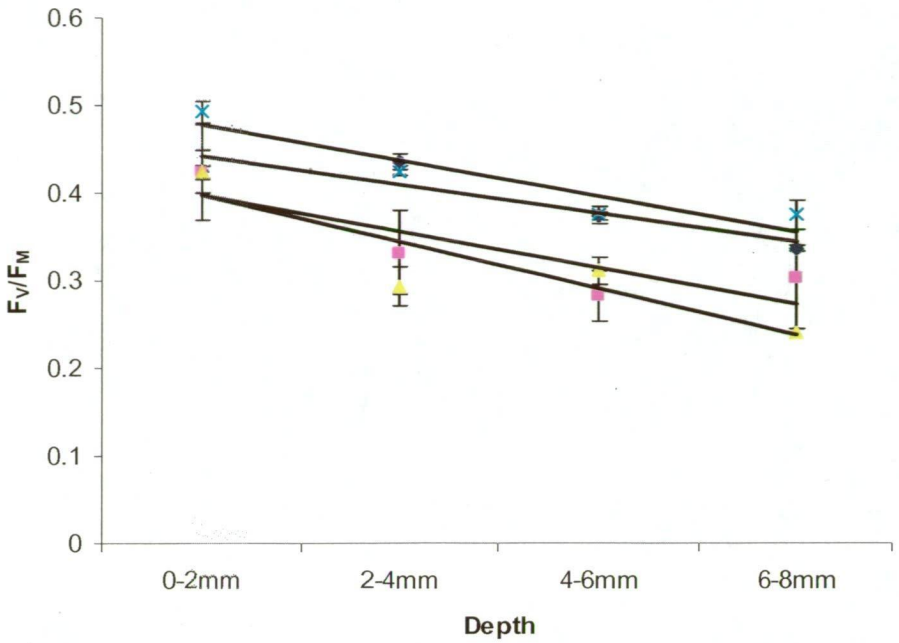


Figure 7. F_v/F_m values by month for the surface 2 mm at Pipe Clay Lagoon (black) and Browns River (red) at sunrise (broken) and midday (solid) from July 2005 to June 2006. Values are means ± Standard Error.

F_V/F_M at both sites declined with depth (Fig. 8). The linear relationship between F_V/F_M and depth in November had an R^2 value of 0.79 to 0.89 in Pipe Clay Lagoon, and an R^2 of 0.89 to 0.98 in Browns River (Table 1.). The November values are representative of the pattern seen in every month of the study. Browns River always had a stronger correlation (R^2) than Pipe Clay Lagoon; this pattern did not change between sunrise and sunset.

a)



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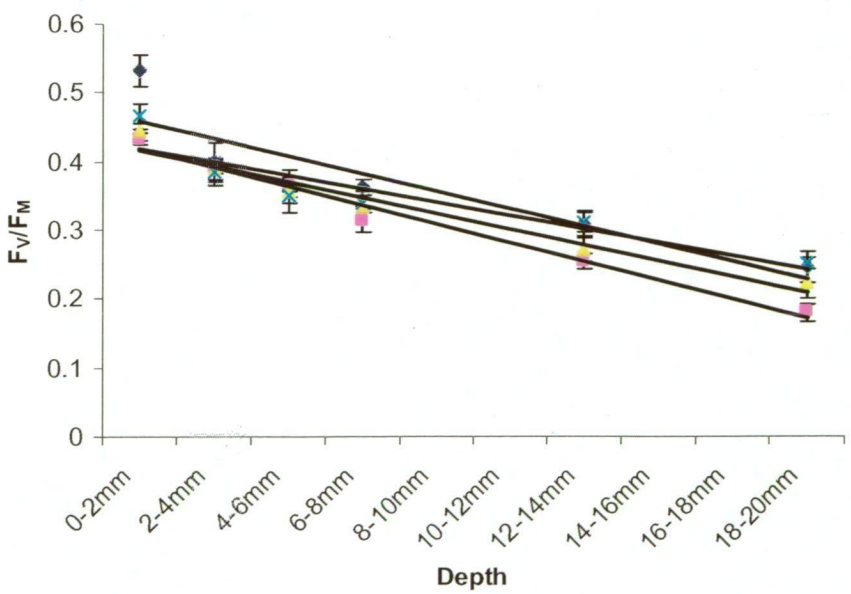


Figure 8. F_V/F_M values through a sediment core taken at four times throughout a day (sunrise= blue, morning= pink, midday= yellow, sunset= green) in November 2005 at a) Pipe Clay Lagoon b) Browns River. Note F_v/F_m between 8-12 mm and 14-18 mm was not measured due to limited time. Values are means ± standard error.

Table 1. R^2 values of F_V/F_M sediment profiles at Pipe Clay Lagoon and Browns River in November 2005.

	Pipe Clay Lagoon	Browns River
Sunrise	0.89	0.89
Morning	0.79	0.97
Midday	0.79	0.99
Sunset	0.87	0.93

The surface rETRmax was relatively low at Pipe Clay Lagoon and ranged from 17.96 ± 0.5 in January to 74.19 ± 9.2 in October (Table 2.). The α readings for Pipe Clay Lagoon were also relatively low, ranging from 0.12 ± 0.01 in July to 0.20 ± 0.01 in November and March. E_k ranged from 98.2 ± 7.2 μMol photons m^{-2} s^{-1} in January to 461.0 ± 55.8 μMol photons m^{-2} s^{-1} in October. The surface rETRmax and E_k were typically higher in spring and early summer with a decrease in late autumn and winter.

Table 2. Rapid Light Curve parameters of microphytobenthos at Pipe Clay Lagoon at sunrise in the surface 2 mm of sediment. Values are means ± standard errors.

Date	$rETR_{max}$	α	E_k (μMol photons m^{-2} s^{-1})
09/07/2005	36.6 ±1.3	0.12 ±0.01	293 ±3.6
27/08/2005	29.0 ±0.9	0.19 ±0.01	155 ±8.5
24/09/2005	34.6 ±0.2	0.19 ±0.01	190 ±15.2
22/10/2005	74.2 ±9.2	0.16 ±0.01	461 ±55.8
12/11/2005	30.8 ±4.2	0.20 ±0.01	158 ±24.9
17/12/2005	24.0 ±1.7	0.18 ±0.01	140 ±14.9
15/01/2006	18.0 ±0.5	0.19 ±0.01	98.2 ±7.2
21/03/2006	33.1 ±3.6	0.20 ±0.01	170 ±20.2
24/04/2006	36.5 ±6.3	0.19 ±0.01	202 ±40.2
20/05/2006	33.1 ±5.1	0.17 ±0.01	206 ±42.0
18/06/2006	36.9 ±1.6	0.16 ±0.01	253 ±0.1

The surface rETRmax values were also relatively low at Browns River, ranging from 22.13 ± 0.9 in July to 65.38 ± 3.9 in October (Table 3.). The surface α values ranged from 0.14 ± 0.01 in November to 0.24 ± 0.01 in September. The E_k values ranged from 126.7 ± 11.2 in June to 416.9 ± 40.7 in November. The surface rETRmax and E_k were typically higher in spring and early summer compared to late autumn and winter.

Table 3. Photosynthetic parameters of microphytobenthos at Browns River at sunrise in the surface 2 mm of sediment. Values are means ± standard errors.

Date	$rETR_{max}$	α	E_k (μMol photons m^{-2} s^{-1})
31/08/2005	53.0 ±6.9	0.17 ±0.01	335 ±59.9
27/09/2005	41.4 ±6.7	0.24 ±0.01	167 ±18.6
18/10/2005	65.4 ±3.9	0.16 ±0.01	319 ±59.6
13/11/2005	56.1 ±4.2	0.14 ±0.01	417 ±40.7
15/12/2005	33.5 ±4.8	0.18 ±0.03	186 ±29.2
17/01/2006	23.9 ±0.3	0.18 ±0.01	137 ±7.4
18/03/2006	29.4 ±1.4	0.20 ±0.01	149 ±4.9
23/04/2006	23.7 ±1.9	0.17 ±0.01	138 ±12.2
27/05/2006	23.9 ±0.3	0.14 ±0.01	186 ±22.7
19/06/2006	27.0 ±2.9	0.21 ±0.01	126 ±11.1

Discussion

Water temperature and salinity were very similar between the two sites investigated in this study, as were pH, dissolved oxygen, turbidity and species composition. Therefore, any difference that was observed between the two sites can most likely be attributed to the differences in sediment grainsize. The nature of the sediment is a major factor determining both the abundance and composition of a community (Cartaxana *et al.* 2006). Some studies report higher biomass in muddy sediment (Riaux-Gobin *et al.* 1987; Perkins *et al.* 2003) while others report a higher biomass level with sandier sediment (Cahoon and Safi 2002). When comparing a sandy (Pipe Clay Lagoon) and muddy (Browns River) site it was observed in this study that the muddier sediment contained a higher biomass. The type of sediment can also affect the depth of light penetration and therefore the size of the euphotic zone (Saburova and Polikarpov 2003). In this study it was observed that the oxygenated/euphotic zone was deeper in the muddier sediment at Browns River compared to the sandy sediments of Pipe Clay Lagoon.

Throughout the study period, the time of day did not appear to significantly affect the biomass of benthic diatoms in the sediment core, as the percentage of chlorophyll *a* in the surface 2 mm and 2-4 mm did not significantly change throughout the day or while comparing the sunrise and midday values at either site throughout the year. The biomass, in the surface 2 mm at the two sites of this study, contained 20-40% of the overall biomass throughout the day. Saburova and Polikarpov (2003) also found approximately 40% of diatoms were present in the surface 2 mm of an intertidal sand flat. If the microphytobenthos were changing position it would be expected that a higher percentage of the algal population would be at the surface at sunrise in comparison to other times during day light. The cells would move towards the sun as it rose but move away in the midday sun to avoid photoinhibition. Perkins (1960) noted, through visual observation that cells remained at the sediment surface while they received light and then migrated down in darkness. Saburova and Polikarpov (2003) also observed that diatoms reached their maximal concentration at the sediment surface during day time emersions. This study examined the sediment profile at two sites for up to 16 hours a day (in summer) during different tide regimes and did not observe upwards or downwards vertical migration; however we did not measure the sediment at night. Vertical migration at the two sites investigated may have been more subtle than the sampling interval used here could detect. For instance, a micro-migration at the surface has been hypothesised to also take place, with individual species cycling within the biofilm at a μm scale rather than mm (Consalvey *et al.* 2004). This study examined the sediment at 2 mm sampling intervals; however Mitbavkar and Anil (2004) observed a high algal biomass in the surface 1 mm of the sediment in summer during morning low tide. Joint *et al.* (1982) also concluded that cells migrate to the top 1 mm during low tide. The 2 mm sampling depth may have been too large to observe these subtle algal movements.

Mitbavkar and Anil (2004) considered that migratory behaviour was not related to time of day, but rather to the duration of light exposure. This implies that it is the time of year that most influences the position of benthic diatoms in the sediment. It has been suggested that in temperate estuaries, microphytobenthic biomass is relatively constant throughout the year (Barranguet *et al.* 1998). In general, neither the abundance nor biomass of the epipelic diatoms shows a marked seasonal variation (Facca and Sfriso

2007). These authors also observed low seasonal variability and concluded that rather than a seasonal scale cell abundance and diversity were affected by a spatial scale. However, Pinckney and Zingmark (1993) found that benthic microalgal biomass generally increased during late winter and early spring, with relatively constant lower levels during late spring and autumn. A seasonal cycle of chlorophyll *a* in biofilm development was observed at the two sites investigated here, with Pipe Clay Lagoon peaking later than Browns River. A higher level of chlorophyll *a* in the surface 2 mm was observed in late spring and summer at Pipe Clay Lagoon and spring in Browns River. This is probably due to the longer day length and warmer water temperatures at this time. It is unclear however, why the biomass at Browns River in summer was so low, with values similar to winter. Wolfstein *et al.* (2000) also observed a decline in summer biomass, at tidal flat stations in the German Wadden Sea, which they concluded was due to benthic invertebrate grazers.

The maximum quantum yield can be used to assess the stress levels of phototrophic biofilm communities. Changes in the value of F_V/F_M can be driven by rapidly reversible mechanisms which can lead to depressed photosynthetic quantum yields or are associated with photodamage and the onset of photoprotective mechanisms (Schofield *et al.* 1998). Seasonal and diurnal changes of F_V/F_M over the study period due to the changing light conditions would be expected. The F_V/F_M values were consistently lower at midday than sunrise between the sites and throughout the year. This pattern of F_V/F_M suggests a down regulation of PSII in response to ambient light conditions rather than damage. Browns River had a significant seasonal pattern with the surface F_V/F_M lower in summer than winter whereas Pipe Clay Lagoon was relatively constant throughout the year, although F_V/F_M was not significantly different between the sites. Browns River had higher F_V/F_M values and lower biomass levels in autumn and winter. This would indicate that the cells were less stressed during winter, perhaps due to the lower light intensity without restricting cold temperature conditions.

The F_V/F_M had a linear relationship with depth with higher values at the surface. This relationship was similar throughout the day and year at the two sites. This is unexpected as the upper most layer of cells are likely to be photoinhibited with the highest F_V/F_M values below the surface. This may be evidence of a micro-migration

taking place or that the sampling interval was too great. A substantial part of the biomass of diatoms, at the two sites studied, may also be found at greater depths due to high tide turbulence (Mitbavkar and Anil 2004) or bioturbation may be occurring. α, E_k and rETRmax did not change considerably throughout the year or between the two sites.

Microphytobenthic cells are typically adapted to a highly variable light environment with cells at the surface exposed to potentially damaging levels of irradiance during day exposure period but periods of darkness after burial. Therefore microalgae developed both behavioural and photophysiological strategies to cope with high light (Consalvey *et al.* 2004). The results of this study imply that these algal communities may be using photophysiological strategies as well as behavioural strategies. Physiological regulation through non-radiative dissipation of excess energy is an important short term process for the photoprotection of PSII against light induced damage (Lavuad *et al.* 2004). Photoprotective dissipation is attributed to rapid modifications within the LHC of PSII, leading to a non photochemical chlorophyll *a* fluorescence quenching (Lavuad *et al.* 2004). They provide photoprotection by quenching the excited states of chlorophylls and by harvesting and efficient transfer of light energy to chlorophylls (Lohr and Wilhelm 2001).

In temperate latitudes intertidal biofilms can be exposed to high temperatures and irradiances (exceeding 2000 μmol photons $m^{-2}s^{-1}$), in particular when tidal emersion periods correspond with solar maxima in summer (Perkins *et al.* 2001). The estimation of the influence of time of day on MPB is complicated by the changes in weather conditions and tidal height. These values affect the positioning of algae and influence the photosynthetic parameters. Throughout the 12 months a mixture of high and low tides were included in the observations however an emphasis has been placed on the overall effect of time of day and season rather than tidal height. Migration is advantageous for benthic diatoms in a dynamic and unstable environment whether to escape intense light and tidal pressures or to increase illumination for photosynthesis. PAM fluorometry is an effective technique for studying intertidal microalgal communities, due to its ability to measure photosynthetic parameters that explain the physiological state of a community at different times of the day and year. In summary, a seasonal pattern of chlorophyll *a* biofilm development was observed at the two sites, with a later peak in the sandy

sediment compared to the muddier sediment. The chlorophyll *a* peak was sharper in the muddier sediment rather than the more gradual incline then decline observed at Pipe Clay Lagoon. As there was no evidence of photoinhibition with F_V/F_M profile values highest at the surface even though the chlorophyll *a* percentage did not change throughout the day it could be concluded that the sampling measurements were too large to observe a micro-migration. The benthic microalgal community at Browns River and Pipe Clay Lagoon did not clearly demonstrate diurnally vertical migration through the sediment although F_V/F_M values did change with the cells more stressed at midday while experiencing the highest illumination. It is likely that the MPB at Pipe Clay Lagoon and Browns River are using photoadaptive strategies in conjunction with a small scale vertical migration.

Chapter 3

Diurnal Changes of Photoadaptive Pigments in Microphytobenthos

Abstract

Microphytobenthos need photoadaptive strategies to survive the highly dynamic light environment in which they reside. Xanthophylls can provide photoprotection by cycling between epoxide and de-epoxide forms, dissipating excess light energy as heat. This study examined the xanthophyll cycle in microphytobenthos on a tidally exposed substrate at Browns River. The goal of this work was to examine to whether microphytobenthos at Browns River used the xanthophyll cycle as a physiological defence against photoinhibition during a natural light-dark cycle (day-night). Changes in PAM fluorescence and xanthophyll: chlorophyll *a* suggests that MPB were under physiological stress at noon. The results indicate that the MPB cells exposed to light at the surface migrated deeper into the sediments to replenish the epoxide form of their xanthophylls. Overall the result suggests that MPBs utilise both behavioural and physiological strategies to survive in the dynamic intertidal environment.

Introduction

In intertidal environments many physical parameters can change over a range of time scales, from minutes (tidal current, wind wave, cloud cover) to seasons (temperature, light and sediment properties) (Koh *et al.* 2007). Microphytobenthos (MPB) and other aquatic photoautotrophs are able to adjust their photosynthetic activity, in response to these changes in ambient light, through physiological regulation and behaviour (Serodio *et al.* 2006). Although this control of the amount of light is achieved by individual cells, its effects are observed at the community level through variation in the biofilm biomass present in the photic zone (Serodio *et al.* 2006). Behavioural regulation is the relationship between light and cell position controlled by vertical migration, which optimises light availability whilst avoiding damaging high irradiances (Jesus *et al.* 2006). Physiological regulation includes non photochemical quenching of

energy by diversion of excess light energy away from photosystem reaction centres using processes such as the xanthophyll cycle (Jesus *et al.* 2006).

There are two photoreactions in all oxygenic photoautorpohic organisms, known as Photosystem I (PSI) and Photosystem II (PSII) (Kolber and Falkowski 1993). In PSII water is photochemically oxidised forming O_2 while simultaneously generating electrons and protons (Kolber and Falkowski 1993). Light absorbed by chlorophyll molecules in the the light harvesting complex results in excitation energy that can undergo one of three fates: photochemistry (delivered to PS II and drives photosynthesis), re-emitted as longer wavelength light (fluorescence) or dissipated as heat (non-photochemical quenching) (Maxwell and Johnson 2000). These three energy sinks occur in competition, therefore an increase in one will result in a decrease of energy flowing through the other two (Maxwell and Johnson 2000). PSII is considered to be the most vulnerable part of the photosynthetic apparatus to light induced damage therefore harm to PSII this will often be the first manifestation of photo-stress (Maxwell and Johnson 2000).

Physiological regulation, through non photochemical quenching (NPQ) by the dissipation of excess energy, is an important short term process for the photoprotection of PSII against light induced damage (Lavuad *et al.* 2004). In diatoms, NPQ includes the photoprotective operation of the xanthophyll cycle (qE, energy dependent quenching) and photoinhibitory damage to the photosynthetic apparatus (ql, photoinhibitory quenching) (Cruz and Serodio 2008). The xanthophyll cycle is present in the thylakoid membranes of all higher plants, ferns mosses and several algal groups (Eskling *et al.* 1997). There are two variants, the violaxanthin cycling which is more commonly found in higher plants and the diadinoxanthin (DD) cycle found in some algal groups (Eskling *et al.* 1997). Xanthophylls are carotenoids containing one or more oxygen radicals and are essential for survival and ecological success (Lohr and Wilhelm 2001). They provide photoprotection by quenching the excited states of chlorophylls and by harvesting and efficiently transferring light energy to chlorophylls (Lohr and Wilhelm 2001). Some of the other methods used to safely dissipate excess light energy via nonphotosynthetic quenching include: differential excitation of PSI vs PSII ratio of cyclic to noncyclic electron transport and state transitions, migration of chlorophyll *a* + protein complex from PSII to PSI (Krause and Weiss 1991), PS II reaction centre quenching (Ivanov *et al.*

2003), chloroplast shading (Jeong *et al.* 2002), nitrate reduction and nitrite excretion (hypothesized as very significant for polar diatoms) (Lomas and Gilbert 1999), and photorespiration and reduced carbon (often glycolate) excretion (Wingler *et al.* 2000).

The xanthophylls diadinoxanthin (DD) and diatoxanthin (DT) are found in many groups of microalgae especially the Bacillariophyceae (diatoms), Chrysophyceae, Xanthophyceae and Dinophyceae (Brown *et al.* 1999). The DD cycle found in diatoms, involves a rapid and reversible conversion from DD (one epoxide form) to DT (de-epoxide group) (Muller *et al.* 2001). DD de-epoxidation begins rapidly after the onset of high light and this rate is light intensity dependent (Lavuad *et al.* 2004). The epoxide to de-epoxide cycle can dissipate excess energy through non-radiative pathways, decreasing the transfer of captured excitation energy to the PSII reaction centres and thus limiting the amount of photodamage to the photosynthetic apparatus (Serodio *et al.* 2005). The xanthophyll cycle is an effective quenching mechanism which does not affect the light harvesting efficiency and lessens the cost of synthesizing other carotenoids (Moisan *et al.* 1998). The amount of DT synthesised via the DD cycle is correlated with the level of qE (Muller *et al.* 2001). NPQ and DT are linearly related and if DT is not present, qE can not occur (Lavuad *et al.* 2004). Under more prolonged, severe light stress qE is replaced by a sustained slowly reversible component of NPQ called qI (Muller *et al.* 2001). In this study we plan to measure the content of DT and DD to estimate qE and measure photosynthetic parameters to estimate qI.

High Pressure Liquid Chromatography (HPLC) is a separation technique used for both quantitative and qualitative pigment analysis. This chromatographic technique, which consists of using small diameter columns, fine particle size and rapid flow rate, is now a widespread tool for investigating microalgal taxonomic compositions (Brotas and Plante-Cuny 2003). The use of HPLC in chemotaxonomy is based on the premise that different algal classes have specific signature or marker pigments, for example fucoxanthin and chlorophyll *b* have been recognised as taxonomic marker pigments for Bacillariophyta and Chlorophyta, respectively (Li *et al.* 2002). This separation method also makes it possible to precisely measure chlorophyll *a* which can be used as a reliable index of biomass of microbenthic algae (Brotas and Plante-Cuny 1998). The use of HPLC pigment analysis is now increasingly being applied to MPB with the additional

advantage of achieving a much better discrimination of degraded pigments (Brotas *et al.* 2007) and the reduction of time consuming microscopic observations (Brotas and Plante-Cuny 2003).

Very little work has been done on MPB in intertidal areas of the southern hemisphere. Previous work has questioned the possible use of migration as a behavioural response to changing light conditions at Browns River (chapter 2). In that previous study a seasonal pattern in chlorophyll *a* and a diurnal pattern of F_v/F_m values were observed however, the benthic microalgal community at Browns River did not clearly demonstrate vertical migration through the sediment. In response to these findings the goal of this current work was to examine to what extent microphytobenthos at Browns River use the xanthophyll cycle throughout the day as a physiological defence against photoinhibition.

Materials and Methods

Several strategies potentially used by MPB to survive in the changing light environment throughout a day were examined at Browns River on the 2nd of September 2007. The location and sediment content of this site is documented in chapter 2. Low tides occurred at 5.45 am and 7.00 pm with a high tide at 11.50 am. The weather was slightly overcast for the entire sampling time with an ambient light level of approximately 1450 µmol photons $m^{-2}s^{-1}$. Samples were collected at sunrise, noon and sunset. During each sampling period seven 45 mm diameter Perspex sediment cores were taken from an artificially shaded (50% decrease in irradiance) and non-shaded site covering an area of 1 m x 1 m. The shaded site was erected 30 minutes before sunrise and present for the entire sampling period. Three cores were taken for fluorescence analysis, three for HPLC pigment analysis and one for taxonomic purposes. The core was manually pushed into the sediment and stoppered using a rubber bung then carefully and quickly returned to shore. The sediment profile was covered with water throughout the day. The 45 mm diameter cores were sliced at the surface 2 mm and 5 mm depth below.

The core samples for HPLC analysis were frozen in liquid nitrogen and transported to a laboratory where they were stored at -80°C freezer until analysis. To extract the pigments, the samples were weighed and quantitatively transferred to 50 ml

centrifuge tubes each containing 9 ml of 4 °C 100% acetone. The tubes were vortexed for about 30 seconds and then sonicated in an ice-water bath for 15 minutes in the dark. The samples were then kept in the dark at 4 °C for approximately 15 hours. After this time the tubes were centrifuged and the supernatant from each tube decanted into a separate 25 ml volumetric flask and were stored in the dark at 4 °C. A second extraction was performed on each MPB sample with a resting time of only 3 hours. The samples were again centrifuged and the supernatant of the second extraction was added to the first. Water was added to each flask such that the final extract mixture was 90:10 acetone: water (vol: vol). Each flask was filled to the 25 ml mark with 90% acetone and then filtered through a 0.2 μm membrane filter (Whatman, anatope) prior to analysis by HPLC. A Waters - Alliance high performance liquid chromatography system was used, comprising a 2695XE separations module with column heater and refrigerated autosampler and a 2996 photo-diode array detector. Immediately prior to injection the sample extract was mixed with a buffer solution (90:10 28 mM tetrabutyl ammonium acetate, pH 6.5: methanol) within the sample loop. After injection pigments were separated using a Zorbax Eclipse XDB-C8 stainless steel 150 mm x 4.6 mm ID column with 3.5 μm particle size (Agilent Technologies) and the gradient elution procedure of Van Heukelem and Thomas (2001) with minor modifications. The flow rate was 1.1 mL min^{-1} and the column temperature was 55°C. The separated pigments were detected at 436 nm and identified against standard spectra using Waters Empower software. Concentrations of chlorophyll *a*, chlorophyll *b* and β,β-carotene in sample chromatograms were determined from Sigma standards while all other pigment concentrations were determined from DHI standards (Denmark). Pigment concentrations were calculated as pigment per wet weight of sediment.

Filtered sea water (0.22 μm membrane filter; Pall Supor, New York) was added to the 2, 2 mm samples taken from 3 cores for fluorescence analysis. A pulse amplitude modulated (PAM) fluorometer (Water PAM, Walz, Effeltrich) was used to determine the chlorophyll fluorescence. To calculate the maximum PSII quantum yield (F_v/F_m) the samples were dark adapted for 15 minutes by wrapping the jars in foil and placing them in a dark container. Rapid light curves (RLC) were taken under software control (Wincontrol, Walz) to obtain values for F_v/F_m, NPQ, and E_k (Ralph and Gademann

2005). Red light emitting diodes (LED) provided the actinic light used in the RLC at levels of 0, 58, 88, 131, 204, 304, 435, 608 and 1012 μmol photons $m^{-2}s^{-1}$. E_k is the light saturation parameter, and is calculated from the intercept between the relative electron transport rate (rETR) and α (Falkowski and Raven 2007). The rETR was calculated by multiplying the irradiance by the quantum yield measured at the end of that interval (Genty *et al.* 1989). PAR vs rETR curves were described using the model of Platt *et al.* (1980) using multiple non-linear regression curve fitting techniques on Systat software (v5.2 for Macintosh Systat Inc.).

The xanthophyll cycle pigments DD and DT were normalised against chlorophyll *a* in this current study to improve the discrimination of cycling by removing the bias caused by variation in amount of MPB biomass (Claustre *et al.*1994). Similar to Brown *et al.* (1999) the xanthophyll pool was calculated as diadinoxanthin + diatoxanthin (DD+DT) and normalized to chlorophyll *a* (Chla). The pigment concentrations and fluorescence parameters were analysed for significant change with a three-way ANOVA using SigmaStat (Systat Software Inc.) after checking for normality and homoscedasticity. The null hypothesis of no difference was considered disproven if the probability was < 0.05 (Appendix 3). The level variability around the mean values is represented by the standard error. Pigment data were further analysed using CHEMTAX (Mackey *et al.* 1996) with an input matrix of pigment ratios derived from published literature (Mackey *et al.* 1996).

Results

Chemtax

Pigment analysis using CHEMTAX confirmed the taxonomic composition observed through microscopic work (Table 1). CHEMTAX estimated that MPB at Browns River was dominated by diatoms with 57.9% of the overall community composition. The major diatom genera present determined by microscopy included *Navicula*, *Cocconeis*, *Nitzschia*, *Amphora* and *Pleurosigma* species.

Table 1. The overall taxonomic composition of Browns River on the 2/9/07 using CHEMTAX (Mackey *et al.* 1996).

	Chlorophyte	Chrysophyte	Cryptophyte	Cyanobacteria	Diatoms	Dinoflagellate	Haptophyte	Prasinophyte
Total pigment %	21.7%	2.8%	14.4%	0.12%	57.9%	0.37%	2.4%	0.35%
STD ERR	4.23%	0.81%	8.60%	0.17%	5.55%	0.11%	0.48%	0.92%

Chlorophyll

All chlorophyll pigments in the surface 2 mm and at 5 mm depth ranged from 5.95 ± 0.5 μg/g at depth at sunrise in the non-shaded site to 14.1 ± 0.3 μg/g at sunrise at the surface. In the artificial shaded area the total chlorophyll ranged from 3.77 ± 0.5 at 5 mm depth at sunrise to 10.3 ± 2.4 μg/g at sunset in the surface 2 mm. At both sites the surface total chlorophyll pigments were significantly greater than at 5 mm depth ($P=0.001$) and the chlorophyll *a* concentrations in the non-shaded site were significantly greater than in the artificially shaded site ($P=0.029$).

When comparing the overall amount of chlorophyll measured down the sediment profile there was a significantly greater percentage of chlorophyll *a* found at the surface relative to the percentage at depth ($P<0.001$). The percentage of chlorophyll in the surface 2 mm was significantly lower at noon compared to sunrise and sunset ($P=0.014$) (Fig 1). In addition, there was an inverse relationship with 5 mm depth.

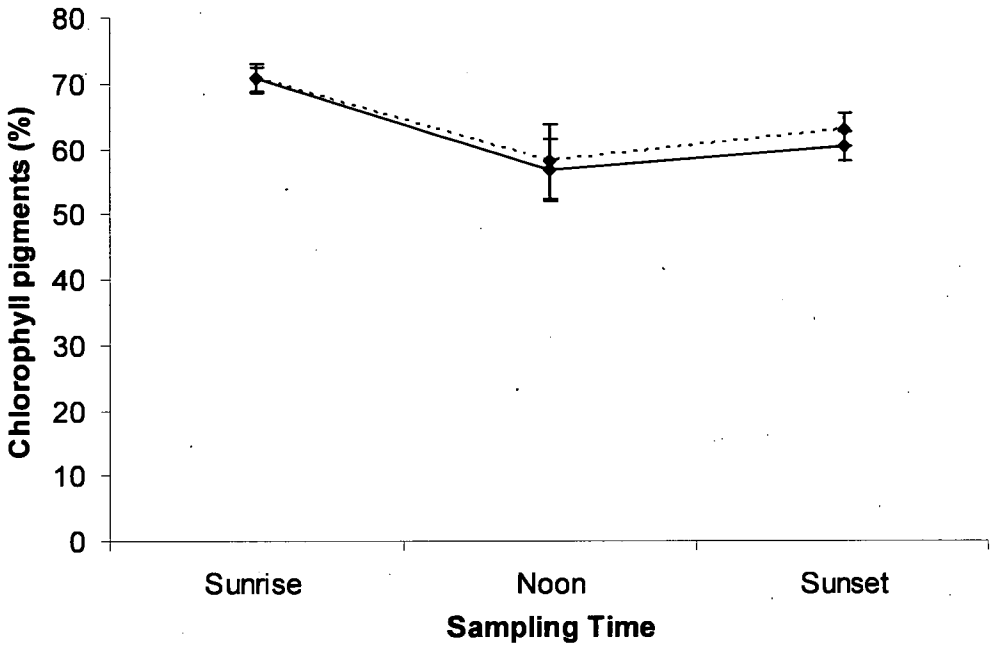
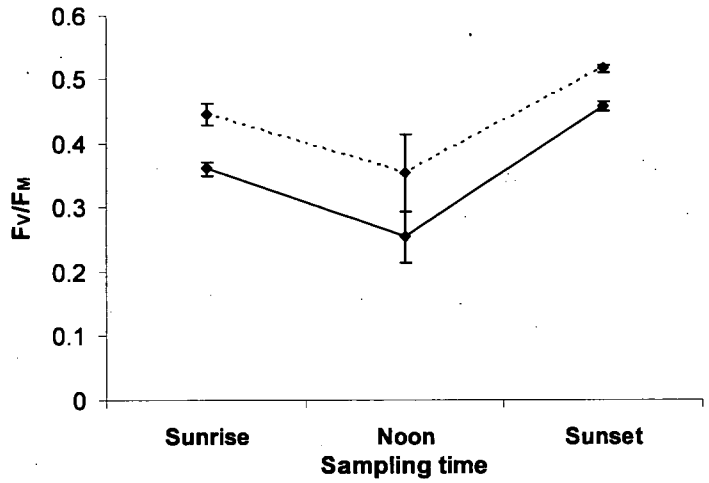


Figure 1. Percentage of total sediment chlorophyll pigments observed at the surface throughout the day on the 2/9/07 at Browns River in a non-shaded (solid) and artificially shaded (broken) site. Values are means ± standard error.

Fluorescence

The maximum PSII quantum yield of chlorophyll fluorescence (F_v/F_m) of the MPBs from the surface 2 mm and at 5 mm depth in a non-shaded area at Browns River ranged from 0.29 ± 0.03 at depth at noon to 0.52 ± 0.01 at the surface at sunrise (Fig 2a). The F_v/F_m value of the surface 2 mm and at 5 mm depth in an artificially shaded area at Browns River ranged from 0.26 ± 0.04 at 5 mm depth at noon to 0.52 ± 0.01 at the surface at sunset (Fig 2b).

a) Non-shaded



b) Artificially shaded

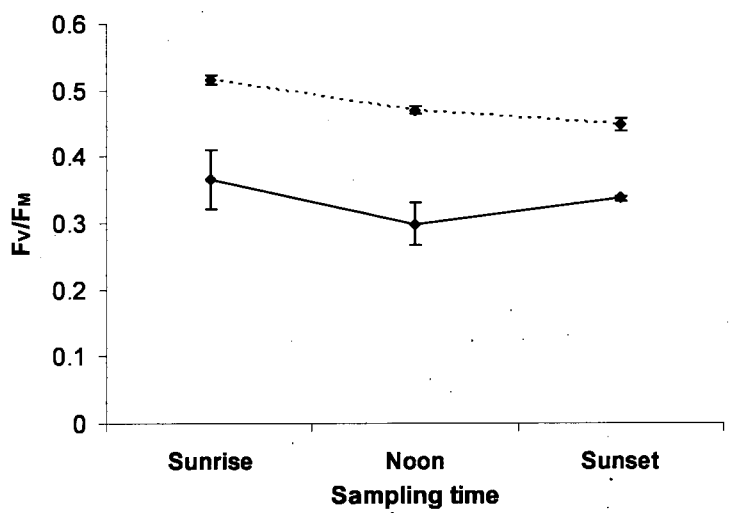
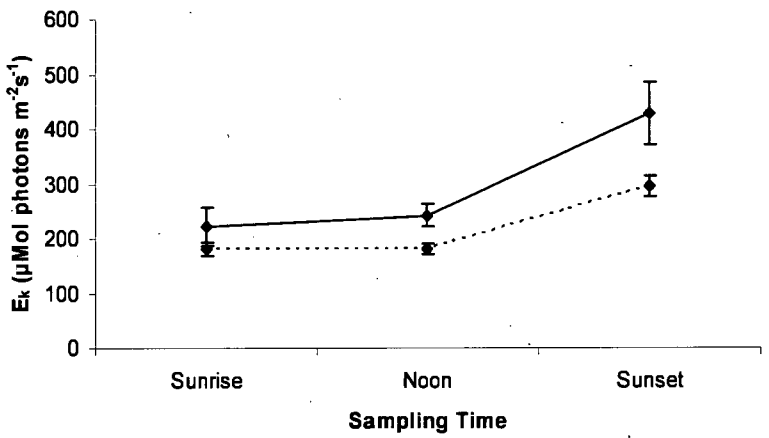


Figure 2. F_V/F_M (broken= surface, solid =5 mm depth) with time at Browns River 2/9/07 in a a) non-shaded area and b) artificially shaded area. Values are means ± standard error.

F_V/F_M values changed significantly throughout the day (P=0.02). Both the shaded and non-shaded sites demonstrated a significant decrease in F_V/F_M at noon compared with sunrise and sunset. The differences between F_V/F_M at the surface and 5 mm depth were significant (P<0.001) with the surface values consistently greater. There was a significant interaction between the time of day and F_V/F_M when comparing the shaded and non-shaded sites (P=0.038).

E_k values in the non-shaded site ranged from 180.9 ± 9.63 μMol photons m^{-2} s^{-1} at the surface at noon to 428.7 ± 57.3 μMol photons m^{-2} s^{-1} at 5 mm depth at sunset (Fig.3a). There was a significant time of day effect with E_k values at sunset greater than sunrise and noon (P=0.04). The values at 5 mm depth were consistently greater than the surface 2 mm values. In the shaded site the E_k values ranged from 231.4 ± 16.5 μMol photons m^{-2} s^{-1} at the surface at noon to 304.9 ± 40.4 μMol photons m^{-2} s^{-1} at the surface at sunrise (Fig. 3b).

a) Non shaded



b) Artificially shaded

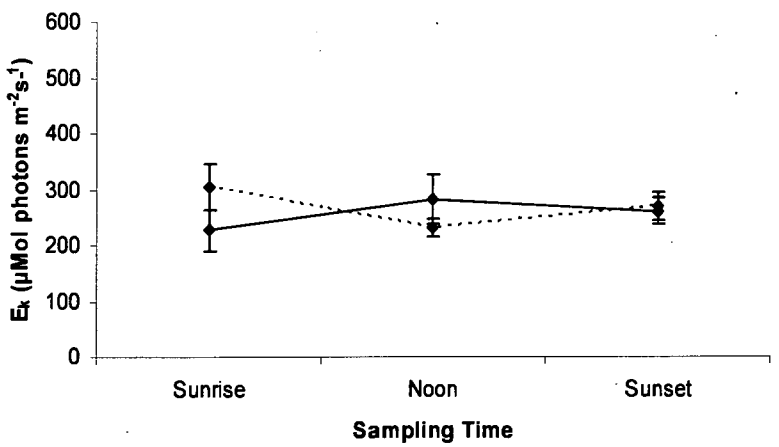
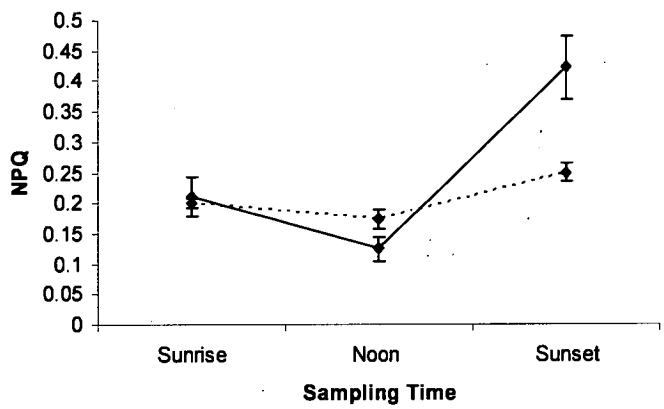


Figure 3. E_k with depth (broken= surface, solid= 5mm depth) and time at Browns River 2/9/07 in a a) non-shaded area and b) artificially shaded area. Values are means ± standard error.

Non-photochemical quenching

Non-photochemical quenching (NPQ) of the benthic microalgae significantly changed throughout the day at Browns River (P=0.008). The NPQ values at the non-shaded site ranged from 0.13 ± 0.02 at noon at 5 mm depth to 0.42 ± 0.05 at sunset at 5 mm depth (Fig. 4a). In the non-shaded site the NPQ values were greater at sunset with 5 mm depth particularly high. At the shaded site the NPQ values ranged from 0.17 ± 0.04 at noon at the surface to 0.32 ± 0.04 at depth at sunrise (Fig. 4b). NPQ at the shaded site was higher at sunrise than noon or sunset. There was not a significant difference between the values of NPQ at the shaded and non-shaded site.

a) Non shaded



b) Artificially shaded

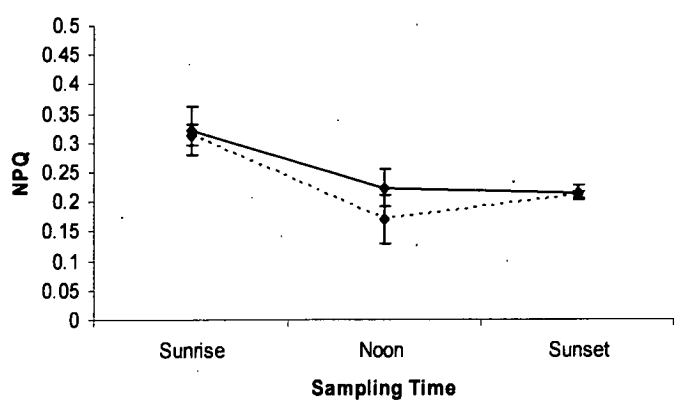


Figure 4. NPQ at E_k with depth (broken= surface, solid= 5 mm depth) and time at Browns River 2/9/07 in a a) non-shaded area and b) artificially shaded area. Values are means ± standard error.

Xanthophyll cycle

The total pigment concentration in the xanthophyll pool normalized to the total chlorophyll *a* (DD+DT/Chl) ranged from 0.072 ± 0.001 μg:μg at the surface at sunset to 0.084 ± 0.005 μg:μg at sunrise at 5 mm depth (Fig 5). The xanthophyll pool pigments were significantly greater at 5 mm depth than at the surface throughout the day (P=0.025). There was no significant difference between sunrise, noon and sunset values or between the shaded and non-shaded sites.

Diadinoxanthin normalised to chlorophyll (DD/Chl) values ranged from 0.038 ± 0.003 μg:μg at 5 mm depth at noon to 0.056 ± 0.004 μg:μg at the surface at sunset in the non-shaded site (Fig 5). In the shaded area DD/Chla ranged from 0.045 ± 0.002 μg:μg at 5 mm depth at sunset to 0.062 ± 0.002 μg:μg at the surface at sunrise. There was a significantly greater mean value of DD/Chla at the surface than at depth (P=0.005). There was a strong tendency for DD/Chla to change with time with a decrease at noon compared to sunrise and sunset (P=0.051).

Diatoxanthin normalised to chlorophyll (DT/Chla) values recorded in the non-shaded site ranged from 0.015 ± 0.004 μg:μg at the surface at sunset to 0.04 ± 0.004 μg:μg at depth at sunrise (Fig. 5). In the shaded site the concentrations ranged from to 0.018 ± 0.01 μg:μg at the surface at noon to 0.04 ± 0.004 μg:μg at sunrise at 5 mm depth. There was a greater amount of DT at 5 mm depth than at the surface (P=0.004). The amount of DT however did not change significantly with time of day or shading.

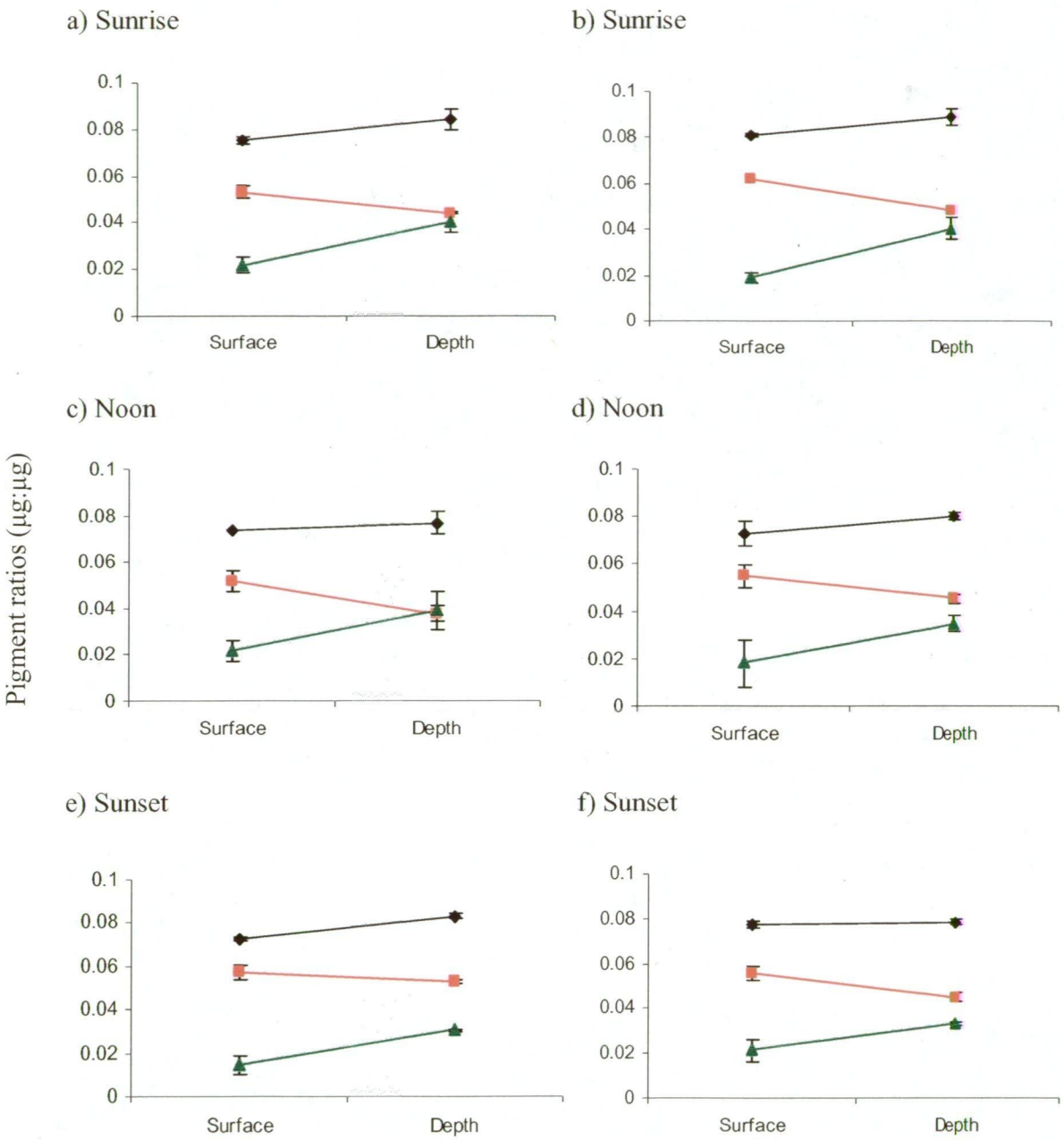
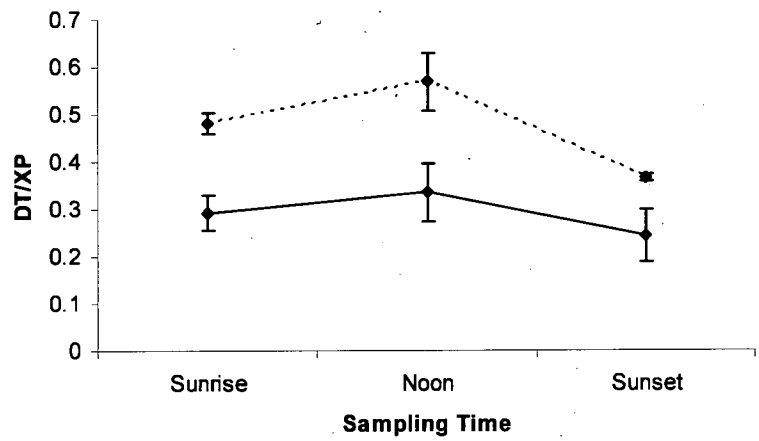


Figure 5. Xanthophyll pigment ratios at Browns River on the 2/9/07 measured in a non-shaded (a,c,e) and artificially shaded site (b,d,f). The xanthophyll pool, diadinoxanthin + diatoxanthin (DD+DT) normalized to chlorophyll *a* (Chla) (black); DD/Chla (red) and DT/Chla (green). Values are means ± standard error.

The amount of DT in relation to the total amount of pigments in the xanthophyll pool (XP) in the non-shaded site ranged from 0.25 ± 0.05 at sunset at the surface to 0.57

± 0.06 at noon at 5 mm depth (Fig. 6). The DT/XP ratio increased at noon compared to sunrise and sunset although this change was not statistically significant. In the shaded area the DT/XP ratios ranged from 0.46 ± 0.04 at 5 mm depth at sunset to 0.23 ± 0.03 at sunrise at the surface. The DT/XP ratio at 5 mm depth remained the same throughout the day in the shaded site however the ratio increased at noon at the surface. The DT/XP ratio was significantly greater at 5 mm depth than the surface (P=0:002).

a) Non shaded



b) Artificially shaded

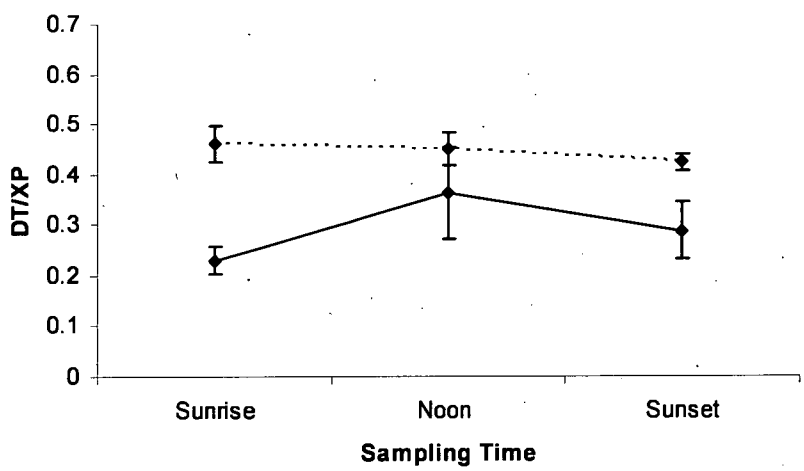


Figure 6. The amount of DT in relation to the xanthophyll pool pigments (DD+DT/Cha) at different times of the day at Browns River on the 2/9/07 (broken= surface, solid= 5 mm depth) measured a a) non-shaded area and b) artificially shaded area. Values are means ± standard error.

Discussion

Although the amplitude and regulation of energy dissipation are species dependent, the following general features can be drawn from previous studies; a fast diadinoxanthin (DD) de-epoxidation and concomitant formation of non-photochemical quenching (NPQ) occurs within seconds, a direct linear relationship is seen between diatoxanthin (DT) accumulation and NPQ development, a *de novo* synthesis of DT accounts for supplementary photoprotection under prolonged illumination, and all parameters (especially those concerning the xanthophyll cycle) are regulated by the light regime (Lavuad *et al.* 2004). Serodio *et al.* (2005) observed that variations in NPQ, upon changes in irradiance, were generally followed by proportional variations in DT content. This was observed under high light, during which the build up of NPQ was closely paralleled by a proportional increase in DT concentration. In diatoms, higher growth irradiances induced larger DD pools, increasing the production of energy dissipating DT under high light and enabling higher NPQ levels (Cruz and Serodio 2008). However, higher NPQ values may also be caused by an increase in photoinhibitory damages to the photosynthetic apparatus (qI) (Cruz and Serodio 2008).

The observations of the relationship between NPQ and DT accumulation at Browns River differed from other findings. An overall linear relationship of NPQ with DT and an inverse relationship with F_V/F_M would be expected, however this was not the case, with DT, NPQ and F_V/F_M all decreasing at noon. An inverse relationship was observed however, between F_V/F_M and NPQ at sunset at the shaded site where F_V/F_M increased and NPQ decreased. This latter response is predictable as there is no need for excess light energy to be quenched. DT and NPQ were both higher at depth than at the surface except at noon in the non-shaded site. It is unclear why DT and NPQ would be higher at depth, where there is very little light compared to the surface. Although, as observed by Serodio *et al.* (2005), development of NPQ capacity in the dark is a form of maintenance to prevent degradation of xanthophyll cycle pigments during prolonged periods of darkness, and providing functional photoprotection upon re-illumination (Serodio *et al.* 2005). Formation of NPQ capability in the dark has been reported for diatoms, being attributed to the conversion of DD to DT as a response to the

establishment of a transthylakoidal pH- gradient, due to chlororespiration or reverse ATP synthase operation (Cruz and Serodio 2008). Further examination of the formation of NPQ capacity in the dark is needed; it has been observed in diatoms however it is unclear how many species undertake this maintenance. Although the majority of cells at Browns River were diatoms (58%) the other groups may exhibit other photoadaptive strategies. Perhaps, as Fujiki *et al.* (2003) observed, the cells are responding to light exposure from the previous day as light history can also regulate the xanthophyll cycle.

Throughout the day at Browns River there was a greater amount of chlorophyll *a* per unit wet weight of sediment at the surface (0-2 mm) than at 5 mm below. This would indicate that there were greater numbers of microalgae at the surface (Brotas and Plante-Cuny 1998). Photosynthesis in intertidal environments is limited to the euphotic zone, the narrow illuminated layer of the surface (Kelly *et al.* 2001). The depth of light penetration and therefore the size of the euphoic zone is affected by the type of sediment. In mud flats, species are generally limited to the upper most mm, with 90% of the light attenuated in the top 400 μm therefore restricting photosynthetic activity (Consalvey *et al.* 2004). At 5 mm depth below the surface very little light would penetrate through the muddy sediment at Browns River, therefore microalgae found at this depth are not actively photosynthesizing. They may not need high levels of light and may have recently migrated there from the surface or are dead/dying. There was a significant decrease in biomass at the surface at noon compared to sunrise and sunset suggesting that the microalgal cells have migrated downward to avoid the potentially damaging light. Migration is also seen as an advantage as it offers safety from tidal currents, reduces disturbance and grazing and increases nutrient availability (Decho 2000). A substantial amount of biomass may also be found deep in sediments, where there is little light penetration.

The chlorophyll *a* concentration was significantly different between the shaded and non-shaded sites, with greater concentrations per gram sediment and therefore more MPB biomass in the non-shaded site. However this result more likely reflects the composition that was present at the randomly chosen sites rather than a treatment effect. Shading the surface of the sediment artificially from 2-55% ambient light during low tide has been shown to increase upward migration of MPB to the surface suggesting that

bright sunlight can inhibit upward migration (Kingston 1999). The shade cloth provided a 50% decrease in light at the shaded site throughout the day; however the E_k values obtained during sampling indicated that the cells at both the shaded and non-shaded sites were saturated at irradiances approximately half that of the ambient light experienced at this time of year.

The F_V/F_M values were on average 25% greater at the surface than 5 mm below. The maximum quantum yield is obtained when all reaction centres are open and is proportional to the fraction of reaction centres capable of converting absorbed light to photochemical energy (Falkowski and Kolber 1993). Thus a low F_V/F_M is potentially an indictor of stress or active photosynthesis. At Browns River the F_V/F_M decreased 20% at noon in both the surface and at 5 mm depth, indicating that the cells were less quenched during this time. This decrease corresponded with a decrease in biomass at this time, indicating that the cells were probably 'stressed' and were moving away from the sunlight. Schofield *et al.* (1998) observed that during times of maximum solar irradiance F_V/F_M of macroalgae decreased by 60% of pre-dawn values but quickly recovered to the higher pre-dawn values within hours after sunset.

The present study was undertaken at the end of winter when water temperatures were still relatively cold (13°C, range over 12 months is 9-21°C see chapter 2). Seasonal changes in NPQ capability may be expected in association with variation in the pool of xanthophyll cycle pigments, since it has been shown that the xanthophyll pool size increases as a response to lower growth temperatures in both diatoms and higher plants (Serodio *et al.* 2005). The xanthophyll cycle is an enzyme mediated reaction and therefore temperature will affect the turnover time of the DD ⇄ DT conversion (Fujiki *et al.* 2003). The exposure to direct sunlight under low temperatures can be particularly damaging to the photosynthetic apparatus as low temperature will slow down the photoprotective response under high light (Serodio *et al.* 2005). An increase in the content of the xanthophyll cycle pigments in winter would thus be an advantage for MPB cells, enhancing photoprotection by allowing higher degrees of de-epoxidation at low temperatures (Serodio *et al.* 2005).

It was observed at Browns River that the xanthophyll pool (DT+DD)/chla did not change throughout the day, although there were greater values at depth than at the

surface. In phytoplankton cells, conversions between DD and DT occurs at a rapid rate (seconds to minutes) but the sum of DD and DT is recognized to remain unchanged on such a small time scale (Fujiki *et al.* 2003). Phytoplankton assemblages have been observed to adjust the xanthophyll pool to variations in ambient irradiance on a time scale of days (Fujiki *et al.* 2003). It is interesting to note that at Browns River the size of the xanthophyll pool at 5 mm depth was greater rather than the surface, although very little light would be expected to penetrate that far. The reason for this observation is unclear. It is possible that photoprotective pigments are being stored in preparation for a sudden exposure to supra-optimal light. Alternatively the cells may have moved down after a high light day the day before or the xanthophyll pool has been diminished on the surface. Fujiki *et al.* (2003) observed that phytoplankton assemblages regulated the xanthophyll cycle within a day depending on the irradiance of the previous day. It is also possible that the cells are migrating up and down within the sediment at a faster time scale than was being detected and therefore the data reflects an average distribution and not that of individual cells.

Greater DT/Chla values were observed at 5 mm depth below the surface where there was little light penetration than the surface. However, DD/Chla was always greater at the surface than at depth. Given excess irradiance, such as a low to high light transition, DD is de-epoxidised into DT and DT accumulates during protracted high light conditions (Moisan *et al.* 1998). In contrast, DT is epoxidised to DD during high to low light transitions and DD accumulates under low light conditions. Kashino and Kudoh (2003) observed that when dark adapted cells were exposed to high light (200 μmol photons $m^{-2}s^{-1}$), followed by exposure to dark, the DD-cycle pigments showed dynamic changes immediately after the change of light conditions, with an increase in DT and decrease in DD. As there was a difference in the DD/Chla and DT/Chla at Browns River at the surface and at 5 mm depth and as DT/Chla concentrations were greater at depth where there was very little light, xanthophyll cycling was occurring within the MPBs migrating up and down in these sediments. At the surface the cells may use up all the DD pigment in the xanthophyll pool then migrate down to depth to replenish this pool. Moisan *et al.* (1998) observed that rapid changes in DT:DD occurred within 5 minutes of the initial irradiance shift. They hypothesized that xanthophyll cycling may help to

optimise photosynthesis in fluctuating light environments which change on minute to hourly time scales (Moisan *et al.* 1998).

The rise in DT/XP at noon indicates that DD is being converted to DT during the highest irradiance of the day. As the cells are likely to be moving vertically as well as activating the DD/DT cycle, this may suggest the rate of vertical movement is no longer sufficient to allow the cells at the surface to have as much DD as they need for photoprotection. Brown *et al.* (1999) similarly observed fluctuations in the molar ratio of DT to the total xanthophyll pool in coral with time. The ratio was lowest at dusk and dawn with its highest values at midday. It was also observed that F_V/F_M decreased with increasing irradiance, reaching a minimum during the period of highest irradiance and subsequently recovering by the next morning (Brown *et al.* 1999). The F_V/F_M values of MPB at Browns River followed this trend of lower values at midday along with greater values of DT/XP. Together these physiological acclimations indicate that the MPB at Browns River use the xanthophyll cycle throughout the day to mitigate excess irradiance and raise photosynthetic performance.

The significant decrease in biomass at the surface at noon compared to sunrise and sunset indicates that the microalgal cells have migrated downward to avoid the potentially damaging light. The lower F_V/F_M values at midday and higher values of DT suggest that at Browns River the xanthophyll cycle is also being used throughout the day to aid acclimation. Therefore MPB at Browns River are utilising both behavioural and physiological strategies to survive in the dynamic changing intertidal environment. The data suggests that the cells exposed to light at the surface are migrating down to replenish the photoadaptive pigments of the xanthophyll pool. The diatoms may use this vertical migration to replenish DT and then remain longer at the surface undergoing high rates of photosynthesis. In combination with access to more nutrients at depth this vertical movement may significantly enhance growth relative to cells at either extreme.

Chapter 4

Discussion and Conclusions

Outcomes

Intertidal areas are important ecosystems found world-wide. They contain high biomass consisting of microphytobenthos (MPB) communities that play a key role in the ecosystem's food webs, add to the stability of the sediment and significantly contribute to productivity (Consalvey *et al.* 2004). This study examined two intertidal sites, Browns River and Pipe Clay Lagoon, in southern Tasmania where very little work has been previously undertaken. MPB have developed photoadaptive strategies to help adjust with the dynamically changing light environment of the intertidal zone. PAM fluorometry and HPLC pigment analysis are effective techniques for studying intertidal communities. Their ability to produce data on photosynthetic parameters and pigments has given valuable insight into the photoadaptive strategies, both behavioural and physiological, used by MPB over short and longer time scales.

A major factor determining both the abundance and composition of benthic communities in intertidal areas is the nature of the sediment (Cartaxana *et al.* 2006). However, there are inconsistencies in the literature in regard to biomass levels and taxonomic composition of MPB in sandy and muddier sediments. Some studies report higher biomasses in muddy sediment (Montani *et al.* 2003; Perkins *et al.* 2003) while others a higher biomass level in sandier sediment (Cahoon and Safi 2002). According to Perkins *et al.* (2003) chlorophyll *a* in sandy sediments is typically lower as muddier sediments are more favourable for nutrient supply, gas exchange and avoidance of desiccation. In contrast Cahoon and Safi (2002) reported lower biomass associated with muddier sediments due to reduced interstitial space volumes, nutrient fluxes and light penetration. The observations at Browns River and Pipe Clay Lagoon, where the water temperature, salinity, pH, dissolved oxygen, and turbidity were very similar, are in agreement with Perkins *et al.* (2003) as the muddier sediment consistently contained higher levels of biomass. The different sediment types also lead to significantly different seasonal patterns of biomass at the two sites.

Mitbavkar and Anil (2004) suggested that migratory behaviour is related to the duration of light exposure rather than the time of day. This implies that it is the time of year that most influences the position of benthic diatoms in the sediment. A study by Facca and Sfriso (2007) in the Venice Lagoon, North Western Adriatic Sea, did not observe a seasonal cycle of diatoms abundance and succession but rather a distribution on a spatial scale. However, by contrast, a higher level of chlorophyll *a* was observed in the surface 2 mm in late spring and summer at Pipe Clay Lagoon and in spring at Browns River. Chlorophyll *a* concentrations in the Tagus Estuary, Portugal, recorded by Cartaxana *et al.* (2006) varied throughout a two year study period with no clear seasonal pattern. In the sandy sediment, they observed higher concentrations in late winter and spring and lower concentrations in summer. In the muddier sediment there was a less consistent pattern with peak concentrations followed by sharp decreases throughout the year, particularly in spring and autumn (Cartaxana *et al.* 2006). The muddier sediment of Browns River also displayed a large peak of biomass in spring followed by a sharp decrease, whereas in the sandy sediment of Pipe Clay Lagoon the increase and subsequent decrease was more gradual over spring and summer.

A significant diurnal pattern of change in MPB biomass was not observed during the 12 month study in the sediment at Pipe Clay Lagoon or Browns River. However the second year study at Browns River did observe a diurnal chlorophyll effect with a significant decrease in surface biomass at noon compared to sunrise and sunset. The cells would have moved towards the sun as it rose but moved away in the midday sun to avoid photoinhibition. It is unclear why a diurnal effect on chlorophyll was observed during the second study at Browns River and not during the initial 12 month study. As the second study at Browns River was undertaken a year after the first sampling perhaps there is an inter-annual variation of vertical migration. This would indicate that a longer term study is required at this site.

Microphytobenthos need to be able to adjust their photosynthetic capacity in the highly variable light environment of intertidal areas. In summer the irradiance can easily reach damaging levels while in winter the cold temperatures slow the photosynthetic rate, and therefore seasonal and diurnal changes of F_v/F_m over the 12 month study period would be expected. The maximum quantum yield (F_V/F_M) can be used as a measure of

stress on the benthic diatom communities. There was evidence of diurnal changes in F_v/F_m with higher values at sunrise than midday all year at both sites. This was also observed in the second year at Browns River, the F_V/F_M decreased at noon in both the surface and at 5 mm depth. This indicates that the algal cells are less 'stressed' at sunrise as there is enough sunlight to photosynthesise but not enough light to induce photoinhibition. F_V/F_M was relatively constant throughout the year at Pipe Clay Lagoon whereas at Browns River had higher F_V/F_M values and lower biomass levels in autumn and winter. This implies that the cells were less quenched during winter, perhaps due to the lower light intensity.

When measuring F_V/F_M the dark adaptation of a sample acts to oxidise the Q_A and reverse non-photochemical quenching. The relative change in quantum yield of fluorescence reflects the level of Q_A, the relationship between photochemistry and fluorescence is inverse and controlled by the redox state of Q_A (Kolber and Falkowski 1993). In some studies the standard 15 minutes of dark adaptation has been considered as insufficient for the total recovery of photosynthetic efficiency (Barrenguet and Kromkamp 2000; Perkins *et al.* 2001). However this length of dark adaptation is commonly used (Honeywill *et al.* 2002, McMinn *et al.* 2004) as it is thought to be sufficient to result in a stable level of Q_A oxidation but it is not long enough for substantial changes in biomass to grow at the sediment surface (Consalvey *et al.* 2004) or photoacclimation through the production of chlorophyll in the cell.

Although a seasonal pattern in chlorophyll *a* and diurnal F_v/F_m changes were observed at Browns River and Pipe Clay Lagoon, the initial results provided little evidence that migration was being used as a behavioural photoadaptive response to high light and therefore the study changed focus and concentrated on physiological strategies at Browns River. Physiological regulation provides photoprotection of PSII against light induced damage through non-radiative dissipation of excess energy (Lavuad *et al.* 2004). Non photochemical quenching (NPQ) increases protection against high light through the dissipation of excess energy and is closely linked to the operation of the xanthophyll cycle (Van Leeuwe *et al.* 2008). At Browns River the total xanthophyll pool did not change throughout the day and the size of the xanthophyll pool at 5 mm depth was greater than at the surface. This is an unexpected result as photosynthesis in intertidal

environments is limited to the euphotic zone, the narrow illuminated layer of the surface (Kelly *et al.* 2001). Light penetration in sediments is dependent on the sediment granulometry;, it decreases with a decrease in grain size (Gomoiu 1967). A study by Ichimi *et al.* (2008) on the estimation of light penetration in intertidal sediments demonstrated that the depth of 1% irradiance in sediment of predominately 63-125 μm was 0.6 mm. The sediment at Browns River consists of approximately 60% <63 μm. Therefore at 5 mm depth below the surface very little light would penetrate through the muddy sediment at Browns River. A large xanthophyll pool size at depth was also observed in a recent study by Van Leeuwe *et al.* (2008) who suggested this was caused by relatively strong bioturbation by sediment fauna which may have induced continuous mixing of the sediment. This may also explain the observations at Browns River.

The amount and regulation of energy dissipation is species dependent, although generally a fast diadinoxanthin (DD) deepoxidation and concomitant formation of NPQ occurs and all parameters (especially those concerning the xanthophyll cycle) are regulated by the ambient light regime (Lavuad *et al.* 2004). Serodio *et al.* (2005) were the first to measure the xanthophyll pigments and to link the presence of diatoxanthin (DT) to NPQ (Van Leeuwe *et al.* 2008). This association of DT with NPQ was observed under high light, during which the build up of NPQ was closely paralleled by a proportional increase in DT concentration. When exposed to excessive irradiance, the light harvesting pigment DD is converted into the heat dissipating pigment DT in the xanthophyll cycle (Van Leeuwe *et al.* 2008). However higher values of DT were observed at 5 mm depth at Browns River where there was little light penetration than at the surface. The results suggest that cells rapidly migrate from the surface when their supply of DD is exhausted and replenish this at depth before returning to the surface. The F_V/F_M values of MPB at Browns River followed this trend of lower values at midday along with greater values of DT/XP. Together these physiological acclimations indicate that the MPB at Browns River use the xanthophyll cycle throughout the day to mitigate excess irradiance and raise photosynthetic performance.

DT and NPQ values at Browns River were higher at depth than at the sediment surface. It is unclear why these values were higher at depth, where there was very little light compared to the surface. It is possible the cells moved down after experiencing high

light the previous day or that the xanthophyll pool had diminished at the surface. Fujiki *et al.* (2003) observed that phytoplankton assemblages regulated the xanthophyll cycle within a day depending on the irradiance of the previous day. It is possible that the higher values at Browns River were caused by photoprotective pigments being stored in preparation for a sudden exposure to damaging light. The capacity of diatoms to develop NPQ in the dark has been considered an adaptive advantage to prevent degradation of xanthophyll cycle pigments during prolonged periods of darkness (Serodio *et al.* 2005). MPB are frequently subjected to rapid changes in light exposure, including the sudden exposure to high levels of sunlight after withstanding long periods in the dark. This occurs frequently with the ebb and flow of the tides throughout the day and therefore the maintenance of functional xanthophyll cycle pigments by benthic diatoms is advantageous (Serodio *et al.* 2005). Further examination of the formation of NPQ in the dark is needed while it has been observed in diatoms it is unclear how many species undertake this maintenance. Although the majority of cells at Browns River are diatoms some may be more motile than others and other algal divisions may contain different photoadaptive strategies. At a sandy site Van Leeuwe *et al.* (2008) observed that the algal community was dominated by non-motile diatom species which perhaps relied heavily on xanthophyll cycling rather than migration.

Measuring the productivity of MPB has many problems because of the highly dynamic intertidal environment and the changeable conditions which control photosynthesis (Serodio *et al.* 2007). The measurement of primary production of MPB can only be achieved in a comparable scale and all methods have their specific advantages and limitation (Wolfstein *et al.* 2000). This study examined MPB on a time scale relevant to light whereas a study by Wolfstein *et al.* (2000) concentrated on tides sampling an hour before low tide which varied from 7 am to 2.30 pm. The measurements therefore, were carried out at comparable times in the tidal cycle but at different times of the day (Wolfstein *et al.* 2000). Intertidal areas are also difficult to research due to the small vertical scale of the euphotic zone of the sediment and the high horizontal heterogeneity on spatial scales from micrometers to metres (Serodio *et al.* 2007). The 2 mm depth used in the current research to examine the sediment profile may have been too large a measurement interval to clearly see vertical migration. Vertical migration may

also be difficult to detect if the cells are cycling within the sediment on a short time scale, as the data therefore reflects average distributions rather than individuals. Round and Palmer (1966) observed that individual species come to the surface at different times and may arrive in a distinct order and remain for differing times. Species may appear at the sediment for a short period before leaving but may return, showing a bimodal presence at the surface (Round and Palmer 1966).

Photophysiological responses of phytoplankton in the water column vary as a function of irradiance, temperature and nutrient status (Wolfstein *et al.* 2000). This is also the case for MPBs on an intertidal flat which are often exposed to a higher variability in environmental conditions (Wolfstein *et al.* 2000). MPB cells are exposed to high frequency changes in irradiances, with the amplitude depending on their position in the sediment, time of day and tidal state (Van Leeuwe *et al.* 2008). Despite the extremely high irradiances, photoinhibition is rarely recorded (Van Leeuwe *et al.* 2008). As seen here, F_V/F_M values in the sediment increased linearly with depth without evidence of photoinhibition at the surface. The significant decrease in biomass observed at Browns River at the surface at noon indicates that the microalgal cells have used a behavioural response and migrated downward to avoid the potentially damaging light. Jesus *et al.* (2006) described migration as an evolutionary strategy, a way to maximise photosynthesis whilst minimising photo damage. However lower F_V/F_M values at noon and higher values of DT suggest that at Browns River the xanthophyll cycle is also being utilised throughout the day to aid photoadaptation.

MPB at Browns River are utilising both behavioural and physiological strategies to survive in the dynamic changing intertidal environment. The exact amount of energy that is allocated to the xanthophyll cycle or migration will be different taxonomically, as some species do not need photoadaptive ability if they have the ability to hide. In terms of the cellular energy budget, migration may be less costly than photophysiological adaptation due to the relatively 'cheap' mechanism of locomotion (Jesus *et al.* 2006) in comparison to producing photoadaptive pigments. Interestingly, some diatoms have been observed to use migration paths made by larger diatoms which would make it even easier to move (Wenderoth *et al.* 2004). Microphytobenthos use different photoadaptive strategies in the dynamic light environment that are time, location and species specific.

MPB photophysiology, and defence mechanisms in particular, warrant further investigation in this community of algae (Van Leeuwe *et al.* 2008). Although the importance of the xanthophyll pigments in photoprotection has been recognised, very few studies have examined the xanthophyll cycling in MPB communities (Van Leeuwe *et al.* 2008).

Conclusions

This research has demonstrated that MPB biomass changes seasonally in a muddy and sandy sediment site and appear to use photoadaptive pigments diurnally. Diatoms in intertidal areas reside in a stressful environment, with the dynamic nature of light, the threat of being washed away, grazers, desiccation and lack of nutrients. MPB use photoadaptive strategies on seasonal and diurnal time scales to explain their success in the intertidal environment. This research highlights the importance of the photoadaptive strategies of MPB in a changing light environment with particular reference to the need of more than one strategy. This work adds to the body of knowledge to help form a more accurate picture. It underlines the fact that current research contains conflicting results on migration, seasonal behaviour and the differences between sediments. As more work is completed the inconsistency should lesson and a more accurate picture be formed. This is essential to understand the primary production of coastal ecosystems.

Future Research

Further work in intertidal areas is needed, particularly in the southern hemisphere, to lessen the inconsistencies and build on current knowledge. Intertidal sediments are vital as one of the most productive ecosystems on earth and will continue to be a major aspect of aquatic botany studies. Ecosystems are complex with numerous interacting factors; therefore it is difficult to just examine one aspect. Tides, temperature and nutrients all play a role and should be examined more closely. These factors will impact on individual species in different ways therefore close examination of taxonomy is needed to examine species adaptations. Further research on the survival strategies of MPB in the extreme intertidal areas is needed, in particular on the xanthophyll cycle and pigments as little work has been previously undertaken. As photosynthesis is an enzyme

reaction, temperature plays a key role in these environments. Intertidal flats experience large, daily temperature fluctuations during low tide due to heating of the sediment. Therefore temperature must be taken into consideration when investigating benthic diatoms through fluorescence and more work should be undertaken to address this.

Appendix 1

Tidal information at sampling times at Browns River and Pipe Clay Lagoon from July 2005 to June 2006. PCL= Pipe Clay Lagoon; BR= Browns River. Note gaps in data are due to only two tides occurring on that day rather than four.

Sampling dates	Site	Sampling time	Tide time	Tide height (m)	High tide (HT) or Low tide (LT)
09/07/2005	PCL	0745	0451	0.81	LT
		1020	0913	1.73	HT
		1330			
		1545			
27/08/2005	PCL	0645	0348	1.34	HT
		1000	0815	1.26	LT
		1300	1423	1.77	HT
		1540	2209	0.77	LT
31/08/2005	BR	0700	0022	0.82	LT
		1000	0708	1.34	HT
		1230	1011	1.27	LT
		1500	1715	1.72	HT
24/09/2005	PCL	0600	0227	1.38	HT
		0930	0705	1.3	LT
		1300	1256	1.72	HT
		1600	2034	0.71	LT
27/09/2005	BR	0600	0527	1.37	HT
		0930	0901	1.3	LT
		1245	1506	1.63	HT
		1600	2246	0.82	LT
18/10/2005	BR	0610	0259	1.03	LT
		1000	0920	1.67	HT
		1320	1627	0.76	LT
		1700	2246	1.44	HT
22/10/2005	PCL	0620	0225	1.42	HT
		1000	0643	1.37	LT
		1330	1218	1.7	HT
		1730	2006	0.65	LT
12/11/2005	PCL	0545	0600	1.56	HT
		1000	1158	1.01	LT
		1400	1749	1.5	HT
		1800	1159	0.85	LT
13/11/2005	BR	0545	0637	1.62	HT
		1000	1310	0.91	LT
		1400	1901	1.42	HT
		1810	0021	0.97	LT
15/12/2005	BR	0520	0751	1.79	HT
		1000	1603	0.6	LT
		1440			
		1845			

Sampling dates	Site	Sampling time	Tide time	Tide height (m)	High tide (HT) or Low Tide (LT)
17/12/2005	PCL	0530	0911	1.75	HT
		1000	1730	0.62	LT
		1430			
		1830			
15/01/2006	PCL	0545	0847	0.97	HT
		1010	1700	0.32	LT
		1430	0051	0.92	HT
		1840			
17/01/2006	BR	0545	0111	0.93	HT
		1010	0240	0.92	LT
		1430	1040	1.25	HT
		1830	1812	0.38	LT
18/03/2006	BR	0700	0610	0.61	LT
		1030	1222	0.95	HT
		1400	1649	0.76	LT
		1730	2344	1.15	HT
21/03/2006	PCL	0710	0101	1.23	HT
		1035	0845	0.41	LT
		1410	1522	0.95	HT
		1730	1912	0.91	LT
23/04/2006	BR	0650	0303	1.33	HT
		1000	1020	0.3	LT
		1300	1808	1.11	HT
		1600	2205	0.78	LT
24/04/2006	PCL	0645	0412	1.3	HT
		1010	1104	0.35	LT
		1300	1845	1.16	HT
		1550	2315	0.69	LT
20/05/2006	PCL	0715	0042	1.36	HT
		1000	0823	0.3	LT
		1300	1520	1.13	HT
		1530	2004	0.91	LT
27/05/2006	BR	0720	0252	0.28	LT
		1010	0941	1.02	HT
		1245	1216	0.97	LT
		1530	1946	1.52	HT
18/06/2006	PCL	0730	0039	1.32	HT
		1000	0758	0.38	LT
		1230	1440	1.23	HT
		1500	2019	0.83	LT
19/06/2006	BR	0730	0152	1.21	HT
		1000	0839	0.47	LT
		1230	1715	1.3	HT
		1500	2138	0.72	LT

Appendix 2

Summary of statistical analysis of chapter 2 of chlorophyll *a* and F_V/F_M at Pipe Clay Lagoon and Browns River (site) over 12 months (season) and time of day (TOD).

Generalised least squares design fit to data using Restricted Likelihood method

Chlorophyll *a*	**DF**	**F**	**P-value**
Site	1	21.995	<0.0001
Season	3	27.140	<0.0001
Site:season	3	9.252	<0.0001
TOD	1	0.210	0.648
Site:TOD	1	0.087	0.769
Season:TOD	3	0.307	0.820
Site:Season:TOD	3	0.866	0.461

F_V/F_M	**DF**	**F**	**P-value**
Site	1	2.680	0.104
Season	3	3.850	0.011
Site:season	3	7.314	0.0002
TOD	1	29.857	<0.0001
Site:TOD	1	0.928	0.337
Season:TOD	3	0.388	0.762
Site:Season:TOD	3	0.963	0.413

Appendix 3

Summary of statistical analysis of chapter 3 of pigment ratios and photosynthetic parameters at Browns River; comparing the surface 2 mm to 5 mm depth (Depth) and the time of day (TOD) in an artificially shaded or non-shaded site (Light).

Data was analysed by a 3-way ANOVA.

Total Chlorophyll*	**DF**	**F**	**P-value**
Depth	1	13.776	0.001
TOD	2	0.398	0.676
Light	1	5.380	0.029

* failed Kolmogorov- Smirnov test for normality

Chlorophyll %	**DF**	**F**	**P-value**
Depth	1	54.534	<0.001
TOD	2	0.291	0.750
Light	1	0.031	0.862

F_V/F_M	**DF**	**F**	**P-value**
Depth	1	17.290	<0.001
TOD	2	4.691	0.019
Light	1	0.067	0.798

E_k	**DF**	**F**	**P-value**
Depth	1	1.663	0.209
TOD	2	3.431	0.049
Light	1	0.073	0.788

NPQ	**DF**	**F**	**P-value**
Depth	1	1.549	0.225
TOD	2	5.856	0.008
Light	1	0.591	0.449

DD+DT	**DF**	**F**	**P-value**
Depth	1	5.703	0.025
TOD	2	1.065	0.360
Light	1	0.588	0.451

DT/chl	**DF**	**F**	**P-value**
Depth	1	9.912	0.004
TOD	2	1.166	0.329
Light	1	0.004	0.947

DD/chl	DF	F	P-value
Depth	1	9.648	0.005
TOD	2	3.372	0.051
Light	1	0.780	0.386

DT/XP	DF	F	P-value
Depth	1	12.097	0.002
TOD	2	1.448	0.255
Light	1	0.082	0.777

Missing data

As there was one value that was missing in the replicates of the PAM parameters a series of 1-way non-parametric ANOVAs were run to see if any of the factors (depth, time, and light) were significant by themselves. If there was a difference an average value was used to replace the missing data and a 3-way ANOVA was run. In all the cases the statistical analysis was consistent; if the difference was significant as a single factor it was also significant in a 3-way ANOVA.

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